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L-Type Calcium Channels in Growth Plate Chondrocytes Participate in Endochondral Ossification

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Abstract Longitudinal bone growth occurs by a process called endochondral ossification that includes chondrocyte proliferation, differentiation, and apoptosis. Recent studies have suggested a regulatory role for intracellular Ca^{2+} (Ca_i^{2+}) in this process. Indirect studies, using Ca^{2+} channel blockers and measurement of Ca_i^{2+}, have provided evidence for the existence of Ca^{2+} channels in growth plate chondrocytes. Furthermore, voltage-gated Ca^{2+} channels (VGCC), and specifically L- and T-type VGCCs, have been recently described in murine embryonic growth plates. Our aim was to assess the effect of L-type Ca^{2+} channel blockers on endochondral ossification in an organ culture. We used cultures of fetal rat metatarsal rudiments at 20 days post gestational age, with the addition of the L-type Ca^{2+} channel blockers verapamil (10–100 μM) or diltiazem (10–200 μM) to the culture medium. Longitudinal bone growth, chondrocyte differentiation (number of hypertrophic chondrocytes), and cell proliferation (incorporation of tritiated thymidine) were measured. Verapamil dose-dependently decreased growth, the number of hypertrophic chondrocytes, and cell proliferation, at concentrations of 10–100 μM. Growth and the number of hypertrophic chondrocytes decreased significantly with diltiazem at 50–100 μM, and proliferation decreased significantly at concentrations of 10–200 μM. Additionally, there was no increase in apoptosis over physiological levels with either drug. We confirmed the presence of L-type VGCCs in rat rudiments using immunohistochemistry, and showed that the antagonists did not alter the pattern of VGCC expression. In conclusion, our data suggest that L-type Ca^{2+} channel activity in growth plate chondrocytes is necessary for normal longitudinal growth, participating in chondrocyte proliferation and differentiation. J. Cell. Biochem. 101: 389–398, 2007.

Key words: growth plate; calcium channels; bone growth; chondrocytes; cartilage

Longitudinal bone growth occurs by a complex and finely coordinated process called endochondral ossification, which takes place at the growth plate. Chondrogenesis is the result of proliferation of growth plate chondrocytes, followed by hypertrophy and apoptosis [Caplan and Boyan, 1994]. Hypertrophic chondrocytes secrete matrix vesicles with a high calcium content, together with a number of substances that form the cartilaginous matrix. There is a concomitant invasion of bone cell precursors and vessels at the metaphyseal end of the growth plate. This process is regulated by multiple local and systemic factors, including parathyroid hormone related protein (PTHrP), fibroblast growth factors (FGFs), Indian hedgehog (Ihh), bone morphogenetic proteins (BMPs),

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growth hormone (GH), insulin-like growth factor I (IGF-I), estrogens, androgens, glucocorticoids, thyroid hormone, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and its metabolites [van der Eerden et al., 2003].

The level of intracellular calcium (Ca_i^{2+}) increases throughout chondrogenesis. An increase in both cytosolic free Ca^{2+} and total Ca_i^{2+} content has been observed as growth plate chondrocytes undergo hypertrophy [Iannotti et al., 1989; Gunter et al., 1990]. Ca_i^{2+} may play a regulatory role in chondrocyte maturation, as an arrest in chondrocyte maturation was observed in chicken growth plate chondrocyte cultures when Ca_i^{2+} was decreased by the application of extracellular EGTA [Zuscik et al., 2002].

Intracellular Ca^{2+} can increase owing to release from intracellular stores or by mechanisms of regulated calcium entry through the plasma membrane. The latter mechanisms include voltage-gated Ca^{2+} channels (VGCC), receptor-operated, store-operated, and second messenger-operated calcium channels. VGCC subtypes are identified by their electrophysiological and pharmacological characteristics. The more widely studied VGCCs to date are the L-, T-, N-, and P-type [Spedding and Paoletti, 1992].

Several in vitro and in vivo studies provide indirect evidence for a role for different types of VGCCs in growth plate physiology. Two in vivo studies using growing rabbits showed a reduction in the hypertrophic zone with the administration of nifedipine, a dihydropyridine that specifically blocks L-type VGCCs [Messler et al., 1990; Duriez et al., 1993]. A decrease in femoral length was also observed in one of these studies [Duriez et al., 1993]. Verapamil, an L-type Ca^{2+} channel blocker that acts at a different site on the channel, produced similar morphologic changes in rabbit growth plates [Messler et al., 1990].

In vitro studies, on the other hand, have produced contradictory results. In experiments in which mouse embryonic mesenchymal bud chondrocytes were cultured with several VGCC antagonists, differentiation was blocked by nifedipine and verapamil, while mineralization was sensitive to lanthanum [Zimmermann et al., 1994]. Lanthanum is a calcium channel blocker with less specificity for L-type channels. The Ca_i^{2+} response to depolarization with K^+ has been investigated in cultures of growth plate chondrocytes from juvenile chickens. An increase in Ca_i^{2+} that was sensitive to Cd^{2+} but insensitive to the dihydropyridines was found, suggesting the existence of N-type, and not L- or T- type VGCCs [Zuscik et al., 1997].

Recently, the expression of VGCC proteins in mouse limb-bud chondrocytes was confirmed by immunocytochemistry and Western blot techniques, using a non-specific pan α-subunit antibody that recognizes the α subunit of the different types of VGCCs [Shakibaei and Mobasheri, 2003]. More specifically, both L- and T-type VGCC α subunit proteins have been detected in mouse growth plate chondrocytes throughout embryonic skeletal development [Shao et al., 2005].

We hypothesized that L-type VGCC in the cell membrane of growth plate chondrocytes play a role in the regulation of endochondral ossification. To investigate this hypothesis we used a fetal rat metatarsal rudiment culture method, which has been well established as a method that maintains the intercellular interactions amongst chondrocytes [Mancilla et al., 1998]. We cultured the bone rudiments with verapamil or diltiazem, two L-type VGCC blockers which act at distinct sites on the α1 subunit of these channels [Spedding and Paoletti, 1992]. We show here the effects of L-type VGCC activity blockade on bone growth, chondrocyte proliferation and differentiation.

MATERIALS AND METHODS

Organ Culture

The second, third, and fourth metatarsal bone rudiments were dissected from Sprague–Dawley rat fetuses at 20 days post-conception and cultured individually in 24-well plates. Each well contained 0.5 ml MEM (Gibco, Invitrogen Corporation) supplemented with 0.05 mg/ml ascorbic acid (Sigma Aldrich, St Louis, MO), 0.3 mg/ml L-glutamine (Sigma), 1 mM sodium glycerophosphate (Sigma), 0.2% BSA (Sigma), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco). Either verapamil (Sigma) at concentrations of 10–100 μM or diltiazem (Sigma) at concentrations of 10–200 μM were added. Verapamil was dissolved in DMSO and a 0.1% DMSO control was included. Diltiazem was dissolved in distilled water. Plates were incubated in humidified air containing 5% CO_2 at 37°C. Culture medium was changed daily. Animal procedures were approved by the Bioethics Committee for Animal Research of the Faculty of Medicine of the University of Chile.

Measurement of Longitudinal Bone Growth

The length of each bone rudiment was measured daily using a dissecting microscope equipped with an eyepiece micrometer.

Assessment of Cell Proliferation

Cell proliferation was assessed by measuring [^{3}H]-thymidine incorporation into newly synthesized DNA as previously described [Bagi and Miller, 1992]. After 2 days of culture, [^{3}H]-thymidine (Amersham, Arlington Heights, IL; SA, 25 Ci/mmol) was added to the culture medium at a concentration of 5 μCi/ml, and the rudiments were incubated for an additional 3 h. The metatarsals were then washed three times for 10 min each time and solubilized overnight using Soluene-350. Total [^{3}H]-thymidine incorporation was then measured by liquid scintillation counting. Each metatarsal rudiment was treated as an individual sample and assayed once.

Assessment of Cellular Hypertrophy

At the end of the second day of culture, metatarsals were fixed in 10% buffered formalin (Merck) for 24 h. After routine processing the metatarsals were embedded in paraffin, and longitudinal 5 μm sections were stained with hematoxilin Harris and toluidine blue. Hypertrophic cells were defined as having a height along the longitudinal axis greater then 10 μm under light microscopy. The number of hypertrophic chondrocytes was quantitated by a blinded observer. In one experiment using verapamil, at concentrations of 0, 40, and 400 μM, hypertrophy was assessed using the localization of activity of alkaline phosphatase by histochemistry. Ten-micrometer cryostat rudiment sections were treated with 4% formaldehyde in PBS for 10 min at room temperature, rinsed in PBS and then placed in 0.1 M Triethanolamine-HCl, pH 8 for 10 min. They were rinsed in DEPC-treated water and stained for alkaline phosphatase for 3 min at room temperature using the simultaneous-coupling azo dye method [Burstone, 1960]. In the working solution (0.5% *N,N*-dimethylformamide and Tris buffer, pH 9.1), 0.03% naphthol AS phosphate (Sigma) was used as substrate, with 0.1% Fast blueBB salt (Sigma) as the azo dye. Slides were rinsed and then counterstained with 0.25% safranin O (Sigma) for 3 s.

Assessment of Apoptosis

Apoptosis was detected by the TUNEL method. Slides were processed similarly to the samples used for immunohistochemistry. Standard methods for TUNEL were followed using the Apoptag Intergen Kit (Cat. No S71000, Intergen, NY). A positive control was carried out with tonsillar tissue. A negative control was done following the same procedure, omitting the addition of the enzyme TdT.

Immunohistochemical Analysis of L-Type VGCC Expression

Metatarsals were fixed in 10% buffered formalin for 3 h. After routine processing the metatarsals were embedded in paraffin and longitudinal 5 μm sections were placed on slides treated with 3-aminopropyltriethoxysilane. Paraffin was then removed with xylol, followed by ethanol hydration. Some slides underwent antigen retrieval with trypsin or heat in citrated buffer, obtaining the same results as in those processed without antigen retrieval. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min at room temperature. Three percent BSA at room temperature was used to block non-specific binding. The slides were incubated at 4°C overnight with anti Ca_v 1.2 (α1c) antibody (ACC-003 Alomone Labs, Israel) diluted 1:50 in 0.05 M TBS, pH 7.6 with 200 μl/L Tween 20. The LSAB-HRP system (DAKO Corp., CA) followed by the Liquid DAB substrate chromogen system were used to detect and visualize binding. Nuclei were stained with Mayer's hematoxilin. Slides were then dehydrated and mounted on permanent hydrophobic media. Controls consisted of rudiments processed without primary antibody.

Statistics

All data were expressed as the mean ± SEM. Statistical significance was determined by ANOVA and post hoc Student's *t*-test for longitudinal growth and hypertrophy. In the case of proliferation, which was expressed as percentage of control, Kruskal–Wallis ANOVA and Mann–Whitney *U*-test were used.

RESULTS

Longitudinal Bone Growth

Fetal rat metatarsal bone rudiments were cultured for 3 days in serum-free medium

containing 0, 10, 40, or 100 μM verapamil and 10, 50, 100, or 200 μM diltiazem. The control group, in the absence of any calcium channel blocker, grew an average of 117.5 μm/day (Fig. 1). Bones cultured in the presence of verapamil (10–100 μM) and diltiazem (10–200 μM), showed decreased growth in a concentration-dependent manner compared with control bones (Fig. 1A,B). This effect was significant at all verapamil concentrations tested ($P < 0.01$ vs. control for 10 μM, and $P < 0.001$ for higher concentrations). In the case of diltiazem, this effect was significant at concentrations of 50–200 μM ($P < 0.001$ vs. control). To assess whether this decreased growth was due to decreased proliferation, decreased cellular hypertrophy, or increased apoptosis, we analyzed these three components.

Cell Proliferation

To assess chondrocyte proliferation we measured [^{3}H]-thymidine incorporation in metatarsal rudiments after 2 days of culture. As shown in Figure 2A,B, verapamil and diltiazem produced a concentration-dependent decrease in [^{3}H]-thymidine incorporation. This effect was significant for both antagonists at all concentrations used ($P < 0.01$ vs. control).

Cellular Hypertrophy

Histological examination was performed on each metatarsal rudiment after 2 days of culture to quantify the number of hypertrophic chondrocytes per section. Verapamil caused a concentration-dependent decrease in the number of hypertrophic chondrocytes (Figs. 3A and 4A–C). This effect was significant at concentrations of 10–100 μM ($P < 0.01$ vs. control for 10 μM, $P < 0.001$ for higher concentrations). Diltiazem also caused a concentration-dependent decrease in the number of hypertrophic chondrocytes (Figs. 3B and 4D–F). This effect was significant at concentrations of 50–200 μM ($P < 0.05$ vs. control for 50 μM and $P < 0.001$ for higher concentrations). One experiment was done with alkaline phosphatase staining as a marker of hypertrophy using verapamil at concentrations of 0, 40, and 400 μM. We observed a dose-dependent decrease in alkaline phosphatase staining similar to the decrease in number of hypertrophic cells (Fig. 5).

Assessment of Apoptosis

To determine whether the decreased hypertrophy and growth could be due to cell death, we assessed apoptosis in rudiments treated with verapamil and diltiazem. Isolated apoptotic bodies located in the metaphyseal end of the hypertrophic zone were observed in control rudiments using the TUNEL assay (Fig. 6A,D). A decrease in apoptosis was observed in rudiments treated with 10 μM verapamil and diltiazem (Fig. 6B,E), while no apoptotic bodies were observed in bone rudiments treated with the higher concentration of both drugs (Fig. 6C,F).

Immunohistochemical Analysis of L-Type VGCC Expression

To confirm the existence of the L-type VGCC in fetal rat metatarsals we performed

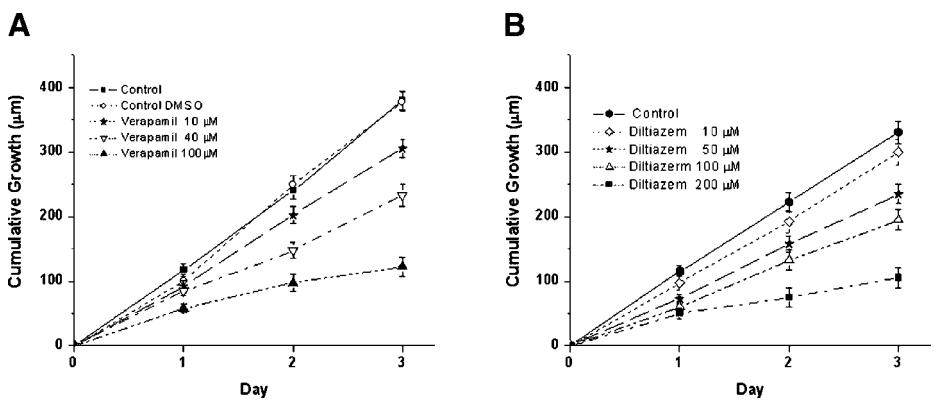

Fig. 1. Longitudinal bone growth. Fetal rat metatarsals were cultured for 3 days in serum-free medium containing 0–100 μM verapamil (n = 40–42 per group, 5 experiments) (**A**) or 0–200 μM diltiazem (n = 13–29 per group, 3 experiments) (**B**). The lengths of the bone rudiments were measured daily using an eyepiece micrometer in a dissecting microscope. Values represent mean ± SEM.

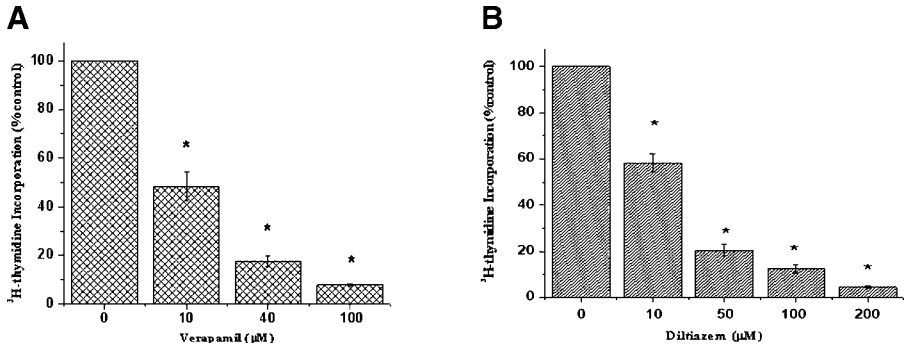

Fig. 2. Total [^{3}H]-thymidine incorporation. Fetal rat metatarsals were cultured for 2 days in serum-free medium containing 0–100 μM verapamil (n = 17–19 per group) (**A**) or 0–200 μM diltiazem (n = 15–18 per group) (**B**). Rudiments were incubated with 5 μCi/ml [^{3}H]-thymidine. Total [^{3}H]-thymidine incorporation was measured by liquid scintillation counting after solubilization. In the case of verapamil the control group (0 μM verapamil) is control vehicle. Values represent mean ± SEM. * $P < 0.01$ versus control.

immunostaining using a specific antibody for the L-type VGCC α-subunit in bone rudiments used as controls. Immunostaining was observed in chondrocytes throughout the rudiment, in the resting, proliferative, and hypertrophic zones (Fig. 7A,C–G,J). No immunostainig was observed in control rudiments treated without the primary antibody (Fig. 7B). Additionally, we analyzed channel protein expression in bone rudiments treated with verapamil and diltiazem. We observed that metatarsals treated with both antagonists presented a distribution of immunostaining for this ion channel that was similar to controls (Fig. 7H,I,K,L).

DISCUSSION

The existing literature confirms the existence of the α subunit of the L- and T-type VGCC in embryonic growth plate chondrocytes, and suggests a regulatory role for calcium in endochondral ossification [Shakibaei and Mobasheri, 2003; Shao et al., 2005]. However, indirect studies are contradictory with regards to the mechanisms responsible for calcium entry in growth plate chondrocytes, and to which type of VGCC may play a role in endochondral ossification [Messler et al., 1990; Duriez et al., 1993; Zimmermann et al., 1994;

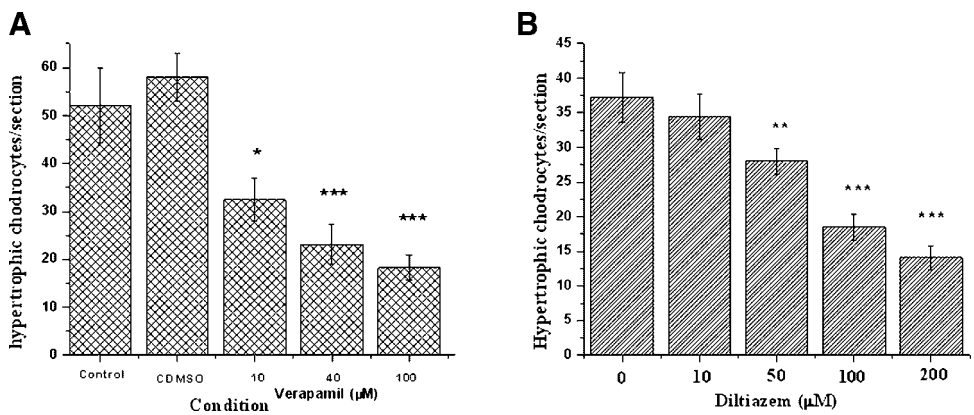

Fig. 3. Quantitation of hypertrophic chondrocytes. Fetal rat metatarsals were cultured for 2 days in serum-free medium containing 0–100 μM verapamil (n = 6–10 per group) (**A**) or 0–200 μM diltiazem (n = 6–8 per group) (**B**). Rudiments were embedded in paraffin and 5 μm sections were obtained. Hypertrophic chondrocytes were operationally defined by a height along the longitudinal axis 10 μm or greater. Values represent mean ± SEM. (CDMSO is control vehicle). $P < 0.01$ versus control, ** $P < 0.05$ versus control, *** $P < 0.001$ versus control.

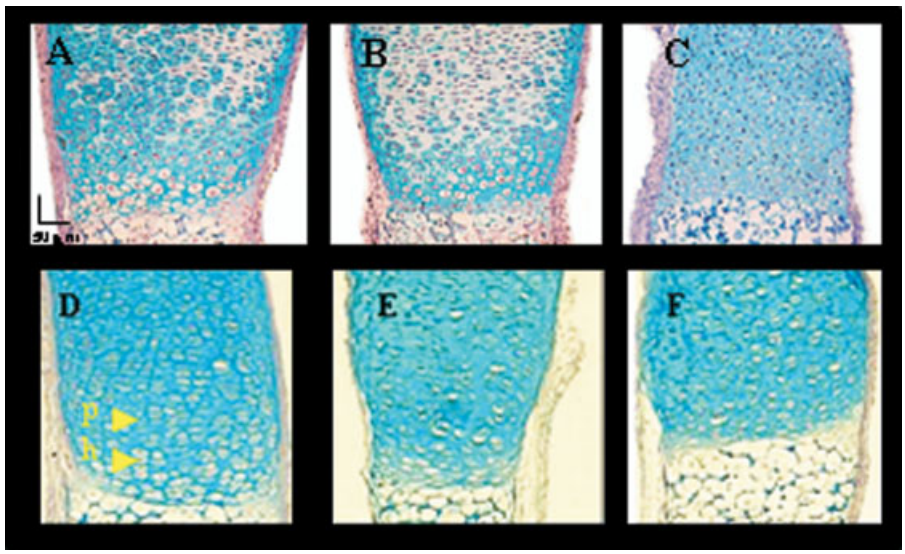

Fig. 4. Representative photomicrographs of fetal metatarsals cultured with verapamil or diltiazem. Bone rudiments were cultured for 2 days in serum-free medium with 0 μM, 10 μM, or 100 μM verapamil (**A–C**) or 0 μM, 10 μM, or 100 μM diltiazem (**D–F**). Representative proliferative and hypertrophic chondrocytes are labeled p and h, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Zuscik et al., 1997, 2002]. In our system of fetal rat metatarsal rudiments in culture, verapamil and diltiazem, two L-type VGCC blockers, produced a dose-dependent decrease in longitudinal bone growth. With this culture method we maintained the interactions amongst chondrocytes at different stages of differentiation, therefore obtaining results that are a better reflection of physiology than studies on isolated chondrocytes. We used two different types of L-type VGCC antagonists, a phenylalkylamine (verapamil) and a benzothiazepine (diltiazem), because these act on distinct sites of the α1 subunit of the channel. We observed a similar, dose-dependent, effect on growth with both drugs, suggesting that the effect is due to channel blockade rather than a non-specific effect of the drug. This is in accordance with a study performed in young rabbits in vivo using nifedipine, another L-type VGCC blocker, where a decrease in humeral growth was noted [Duriez et al., 1993].

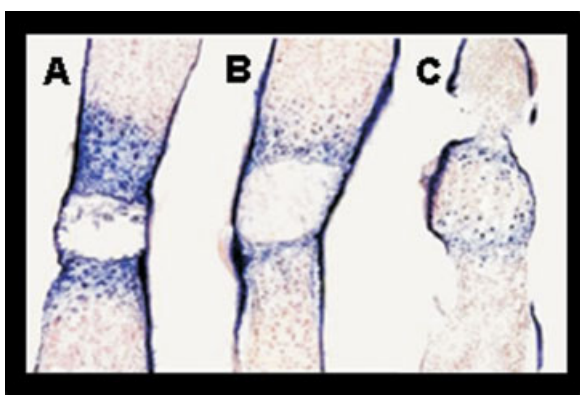

Fig. 5. Photomicrographs demonstrating the effect of verapamil on alkaline phosphatase activity: 0 μM verapamil (**A**), 40 μM verapamil (**B**), 400 μM verapamil (**C**). Samples were embedded in OCT compound, and 10 μm cryostat sections were cut and mounted onto poly-L-lysine-coated slides. Enzyme histochemistry was performed using the simultaneous azo dye method. Alkaline phosphatase is observed as a purple-blue stain. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

To clarify the mechanism by which longitudinal growth may be affected, we assessed two components of bone growth: proliferation and hypertrophy. Both blockers produced a dose-dependent decrease in these two parameters. This is in agreement with an in vitro study using mesenchymal limb bud chondrocyte cultures, in which a decrease in chondrocyte differentiation was observed with nifedipine [Zimmermann et al., 1994]. A decrease in chondrocyte hypertrophy was also observed in vivo in young rabbits administered nifedipine and verapamil [Messler et al., 1990; Duriez et al., 1993]. However, our results contradict those of another study using juvenile chicken growth plate chondrocyte cultures, in which no effect of nifedipine was observed on the depolarization-induced increase in Ca_i^{2+} [Zuscik et al., 1997]. The effect on isolated chondrocytes may

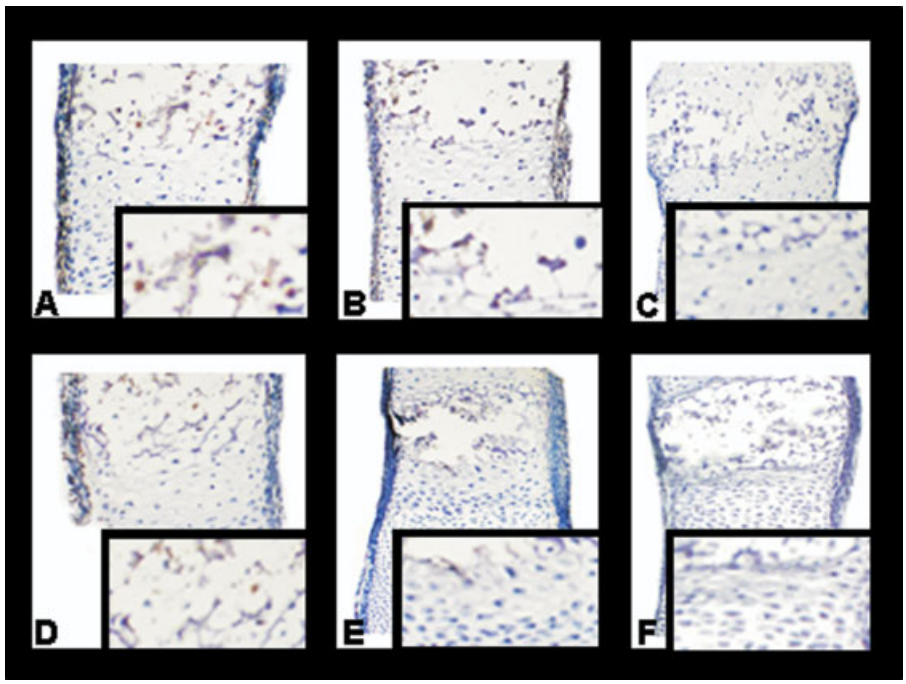

Fig. 6. Photomicrograph showing apoptosis on bone rudiment sections treated with verapamil or diltiazem. Apoptosis was detected by the TUNEL method, and is observed as a brown staining. Fetal rat metatarsals were cultured for 2 days in serum-free medium containing 0 μM (**A**), 10 μM (**B**) or 100 μM verapamil (**C**), or 0 μM (**D**), 10 μM (**E**), or 200 μM diltiazem (**F**). Amplified sections of the metaphyseal end of the hypertrophic zone where the apoptosis is located are shown in each photograph.

be different from that observed in the organ as a whole.

We assessed apoptosis with the TUNEL assay in control rudiments and in rudiments treated with different antagonist doses. We observed apoptosis with this method in control bones at the metaphyseal end of the hypertrophic zone, as has been previously described. Interestingly, we observed a decrease in apoptosis in this zone in bone rudiments treated with either antagonist. Furthermore, there was no histological evidence of cell death. This rules out the possibility of a toxic drug effect causing the decreased growth, proliferation, and differentiation. These results also support the hypothesis that decreased rudiment growth is due to a decrease in proliferation and differentiation, rather than an acceleration of differentiation and apoptosis. The decreased number of hypertrophic chondrocytes observed is not due to increased apoptosis.

It must be kept in mind that the half-maximal inhibitory concentrations of Ca^{2+} channel antagonists vary for different cells and have been described in the range of 0.02–50 μM [Hille, 1992]. Considering that the concentration of the drug that reaches the chondrocyte in an organ culture is unknown, we used concentrations in the higher end of the range used in the incubation of isolated cells. At the lower concentrations used, both of these drugs are specific for L-type calcium channels, while verapamil also blocks T-type channels at intermediate concentrations [Spedding and Paoletti, 1992]. However, higher concentrations probably block other calcium entry pathways, including calcium pumps and channels that may be formed by annexins. The latter is a group of calcium channel-forming proteins that has been described in growth plate chondrocytes [Arispe et al., 1996; Wang and Kirsch, 2002; Wang et al., 2003]. Under our experimental conditions, the effect at the lower and intermediate doses used should reflect a specific effect of L-type channel blockade, while at higher doses inhibition of other calcium channels, including annexin channels, may also be involved.

Several of the hormones involved in the regulation of endochondral ossification have effects on membrane calcium transport in other cell types such as osteoblasts and myoblasts.

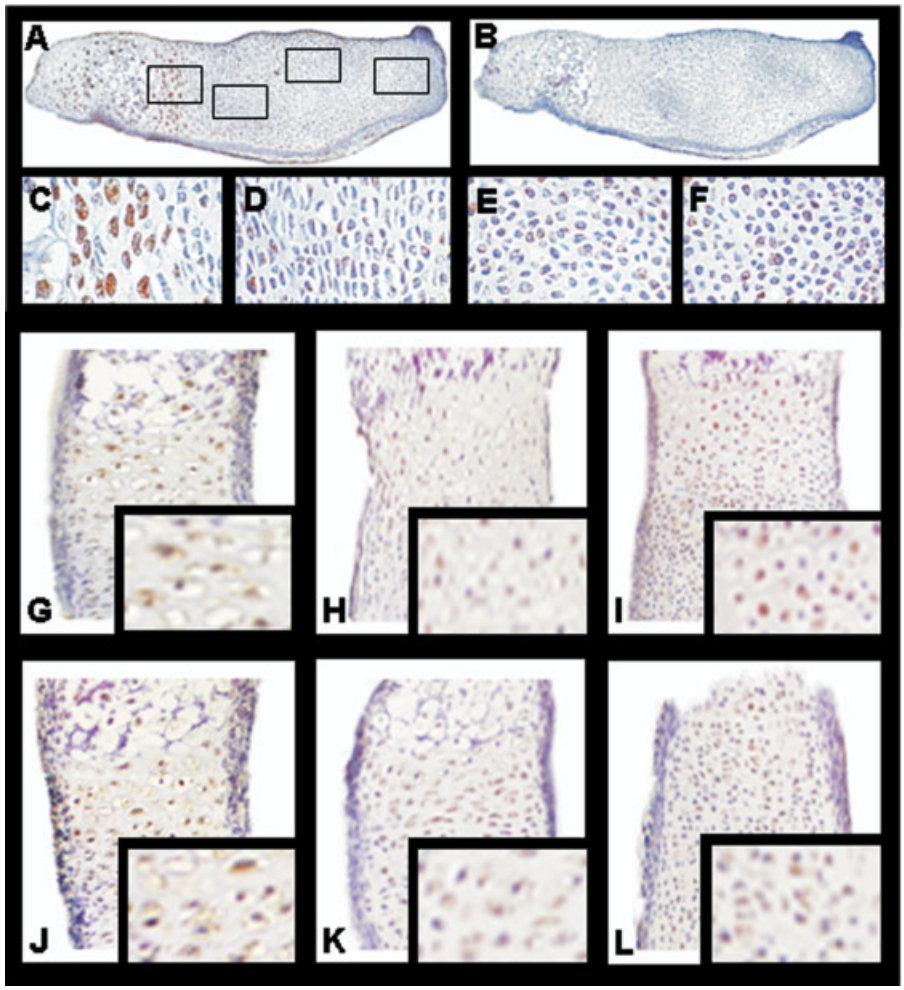

Fig. 7. Photomicrograph showing immunostaining for the α subunit of L-type voltage gated calcium channel (VGCC) in sections of control bone rudiments (**A**, **G**, **J**), and rudiments treated with 10 μM verapamil (**H**), 100 μM verapamil (**I**), 10 μM diltiazem (**K**), or 200 μM diltiazem (**L**). Metatarsals were fixed, embedded in paraffin and longitudinal 5 μm sections were placed on slides that were incubated with anti Ca_v 1.2 (α1c) antibody. Amplified sections of the different maturation zones of the immunostained control bone (**A**) are shown separately. (**C:** hypertrophic, **D:** proliferative, and **E**, **F:** resting zone). A control rudiment treated without primary antibody showing no immunostaining is depicted in **B**.

IGF-I, PTHrP, 1,25 $(OH)_2$ Vitamin D3 and its metabolites have been shown to affect calcium channels [Caffrey and Farach-Carson, 1989; Poiradeau et al., 1997; Solem and Thomas, 1998; Brines and Broadus, 1999; Wang et al., 1999; Liu et al., 2000; Lalonde et al., 2001; Zanello and Norman, 2003; Zheng et al., 2004]. PTHrP has been described as the gatekeeper of chondrocyte maturation, limiting progression to hypertrophy. This effect has been demonstrated in mice lacking or overexpressing the gene for this hormone, and in patients presenting an activating mutation of its receptor [Schipani et al., 1995; Vortkamp et al., 1996; Lanske et al., 1999; Povot and Schiapani, 2005]. In growth plate chondrocytes, it was shown that the PTHrP gene may be regulated by Ca_i^{2+} [Zuscik et al., 2002]. At lower Ca_i^{2+} concentrations there was an increased expression of PTHrP, and a domain in the PTHrP gene was identified that conferred calcium sensitivity to this gene. Therefore, L-type Ca^{2+} channels may be related to local and systemic factors involved in endochondral ossification in two ways; by responding directly or indirectly to these factors and altering calcium entry, or by producing changes in Ca_i^{2+} that affect the synthesis and action of these factors. Further studies are necessary to solve these questions.

L-type Ca^{2+} channels are present in growth plate chondrocytes at all stages of differentiation as shown by our immunohistochemical

analysis. Calcium entry may be differentially regulated at the different maturation stages, and our data suggest that L-type channels are one of the entry pathways for Ca^{2+}. The regulation of chondrogenesis by changes in Ca_i^{2+} may depend, at least in part, on L-type Ca^{2+} channel activity. We conclude that L-type Ca^{2+} channel activity in growth plate chondrocytes is necessary for normal longitudinal growth by participating in chondrocyte proliferation and differentiation.

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REFERENCES

Arispe N, Rojas E, Genge BR, Wu LN, Wuthier RE. 1996. Similarity in calcium channel activity of annexin V and matrix vesicles in planar lipid bilayers. Biophys J 71:1764–1775.

Bagi CM, Miller SC. 1992. Dose-related effects of 1, 25-dihydroxyvitamin D3 on growth, modeling, and morphology of fetal rat metatarsals cultured in serum-free medium. J Bone Miner Res 7:29–40.

Brines MA, Broadus AE. 1999. Parathyroid hormone related protein potentiates depolarization induced release in PC-12 cells via L-type voltage-sensitive Ca^{2+} channels. Endocrinology 140:2646–2651.

Burstone MS. 1960. Histochemical observations on enzymatic processes in bone and teeth. Ann NY Acad Sci 85: 431–444.

Caffrey JM, Farach-Carson MC. 1989. Vitamin D3 metabolites modulate dihydropyridine-sensitive calcium currents in rat osteosarcoma cells. J Biol Chem 264(34): 20265–20274.

Caplan AI, Boyan BD. 1994. Endochondral bone formation: The lineage cascade. In: Hall BK, editor. Bone Volume 8. Mechanisms of bone development and growth. Boca Raton: CRC Press. pp 41–45.

Duriez J, Flautre B, Blary MC, Hardouin P. 1993. Effects of the calcium channel blocker nifedipine on epiphyseal growth plate and bone turnover: A study in rabbit. Calcif Tissue Int 52(2):120–124.

Gunter TE, Zuscik MJ, Puzas JE, Gunter KK, Rosier RN. 1990. Cytosolic free calcium concentrations in avian growth plate chondrocytes. Cell Calcium 11:445–457.

Hille B. 1992. Calcium channels. In: Hille B, editor. Ionic channels of excitable membranes. 2nd edition. Sunderland, Massachusetts: Sinauer Associates, Inc. 108p.

Iannotti JP, Brighton CT, Stambough JE. 1989. Cytosolic ionized calcium concentration in isolated chondrocytes from each zone of the growth plate. J Orthop Res 7:511–518.

Lalonde M, Li B, Kline L, Pang P, Karpinski E. 2001. Modulation of L-type Ca^{2+} channels in UMR 106 cells by parathyroid hormone-related protein. Life Sci 70(5):503–515.

Lanske B, Amling M, Neff L, Guiducci J, Baron R, Kronenberg H. 1999. Ablation of PTHrP gene or PTH/PTHrP receptor gene leads to distinct abnormalities in bone development. J Clin Invest 104:399–407.

Liu R, Li W, Karin NJ, Bergh JJ, Adler-Storthz K, Farach-Carson MC. 2000. Ribozyme ablation demonstrates that the cardiac subtype of voltage sensitive calcium channel is the molecular transducer of 1, 25 dihydroxyvitamin D3 stimulated Ca influx in osteoblast cells. J Biol Chem 275(12):8711–8718.

Mancilla EE, De Luca F, Uyeda JA, Czerwiec FS, Baron J. 1998. Effects of fibroblast growth factor-2 on longitudinal bone growth. Endocrinology 139:2900–2904.

Messler HH, Koch W, Muzenberg KJ. 1990. Analogous effects of organic calcium antagonists and magnesium on the epiphyseal growth plate. Clin Orthop 258:135–141.

Poiradeau S, Lieberherr M, Kergoise N, Corvol MT. 1997. Different mechanisms are involved in intracellular calcium increase by insulin-like growth factors 1 and 2 in articular chondrocytes: Voltage-gated calcium channels, and/or phospholipase C coupled to pertussis-sensitive G protein. J Cell Biochem 64:414–422.

Povot S, Schiapani E. 2005. Molecular mechanisms of endochondral bone development. Biochem Biophysic Res Commun 328:658–665.

Schipani E, Kruse K, Juppner H. 1995. A constitutively active mutant PTH-PTHrP receptor in Jansen metaphyseal chondrodysplasia. Science 268:98–100.

Shakibaei M, Mobasheri A. 2003. β1-integrins co-localize with Na, K-ATPase, epithelial sodium channels (EnaC) and voltage activated calcium channels (VACC) in mechanoreceptor complexes of mouse limb-bud chondrocytes. Histol Histopathol 18:343–351.

Shao Y, Alocknavitch M, Farach-Carson C. 2005. Expression of voltage sensitive calcium channel (VSCC) L-type Cav1.2 (α1c) and T-type (α1H) subunits during mouse development. Dev Dyn 234:54–62.

Solem ML, Thomas AP. 1998. Modulation of cardiac Ca^{2+} channels by IGF-I. Biochem Biophys Res Commun 252:151–155.

Spedding M, Paoletti R. 1992. Classification of calcium channels and the sites of action of drugs modifying channel function. Pharmacological Reviews 44(3):353–375.

Van der Eerden BCJ, Karperien M, Wit JM. 2003. Systemic and local regulation of the growth plate. Endocrine Reviews 24:782–801.

Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ. 1996. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 273:613–622.

Wang W, Kirsch T. 2002. Retinoic acid stimulates annexin-mediated growth plate chondrocyte mineralization. J Cell Biol 157:1061–1070.

Wang ZM, Messi ML, Renganathan M, Delbono O. 1999. Insulin-like growth factor-1 enhances rat skeletal muscle charge movement and L-type Ca^{2+} channel gene expression. J Physiol 516:331–341.

Wang W, Xu J, Kirsch T. 2003. Annexin-mediated Ca^{2+} influx regulates growth plate chondrocyte maturation and apoptosis. J Biol Chem 278:3762–3769.

Zanello LP, Norman AW. 2003. Multiple molecular mechanisms of 1α, 25 (OH)2- vitamin D3 rapid modulation of three ion channel activities in osteoblasts. Bone 33:71–79.

Zheng Z, Wang ZM, Delbono O. 2004. Ca(2+) calmodulin kinase and calcineurin modulate IGF-1 induced skeletal muscle dihydropyridine receptor alpha (1s) transcription. J Membr Biol 197(2):101–112.

Zimmermann B, Lange K, Mertens P, Bernimoulin JP. 1994. Inhibition of chondrogenesis and endochondral mineralization in vitro by different calcium channel blockers. Eur J Cell Biol 63(1):114–121.

Zuscik MJ, Gunter TE, Puzas JE, Rosier RN. 1997. Characterization of voltage-sensitive calcium channels in growth plate chondrocytes. Biochem Biophys Res Comm 234:432–438.

Zuscik MJ, D'Souza M, Gunter KK, Gunter TE, O'Keefe RJ, Schwarz EM, Puzas JE, Rosier RN. 2002. Growth plate chondrocyte maturation is regulated by basal intracellular calcium. Exp Cell Res 276:310–319.