Calcium mediates dorsoventral patterning of mesoderm in *Xenopus*

Verónica Palma*†‡, Manuel Kukuljan† and Roberto Mayor*

Calcium signals participate in the differentiation of electrically excitable and nonexcitable cells; one example of this differentiation is the acquisition of mature neuronal phenotypes [1]. For example, transient elevations of the intracellular calcium concentration have been recorded in the ectoderm of early embryos, and this elevation has been proposed to participate in neural induction [2–5]. Here, we present molecular evidence indicating that voltage-sensitive calcium channels (VSCC) are involved in early developmental processes leading to the establishment of the dorsoventral (D-V) patterning of a vertebrate embryo. We report that α_1S VSCC are expressed selectively in the dorsal marginal zone at the early gastrula stage. The expression of the VSCC correlates with elevated intracellular calcium levels, as evaluated by the fluorescence of the intracellular calcium indicator Fluo-3. Misexpression of VSCC leads to a strong dorsalization of the ventral marginal zone and induction of the secondary axis but no direct neuralization of the ectoderm. Moreover, specific inhibition of VSCC by the use of calcicludine results in ventralization of the dorsal mesoderm. Together, these results indicate that calcium channels regulate mesodermal patterning by specificating the D-V identity of the mesodermal cells. The D-V patterning of the mesoderm has been shown to depend on a gradient of BMPs activity. We discuss the possibility that VSCC affect or act downstream of BMPs activity.

Addresses: *Facultad de Ciencias, Universidad de Chile and Millennium Nucleus in Developmental Biology, Las Palmeras 3425, Santiago, Chile. †Programa de Fisiología y Biofísica, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Av. Independencia 1027, Santiago, Chile.

‡Present address: Skirball Institute of Biomolecular Medicine, New York University Medical Center, 540 First Avenue, New York, New York 10016, USA.

Correspondence: Manuel Kukuljan, Roberto Mayor
E-mail: kukuljan@neuro.med.uchile.cl
rmayor@abello.dic.uchile.cl

Received: **18 May 2001**
Revised: **3 August 2001**
Accepted: **31 August 2001**

Published: **16 October 2001**

Current Biology 2001, 11:1606–1610

0960-9822/01/$ – see front matter

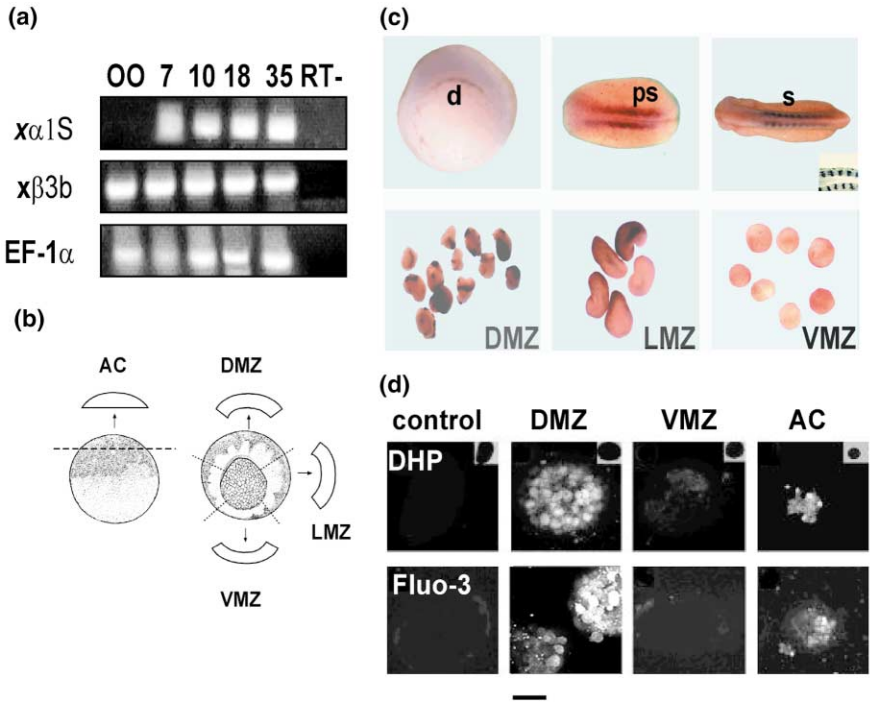
Results and discussion

VSCC are expressed in the dorsal mesoderm during gastrulation

We isolated a cDNA-encoding part of a *Xenopus laevis* that is homologous to the gene that encodes the α_1S main ion-conducting subunit of VSCC (Xα_1S) in other vertebrate species. The corresponding transcript is present early during development, being detectable starting from stage 7, a time at which zygotic transcription starts (Figure 1a). In order to analyze the spatial distribution of this transcript, we performed in situ hybridization of the whole embryo or of mesodermal explants. The in situ hybridization (ISH) signal was undetectable before stage 10, probably because of the lower sensitivity of this technique as compared to the RT-PCR. Analysis by whole-mount ISH of the expression of a previously identified VSCC accessory subunit, Xβ_3 [8], showed a pattern similar to Xα_1S (data not shown). At stage 10, the expression is localized at the dorsal marginal zone (DMZ), but a weak staining was detected in the prospective ectoderm by in situ hybridization and RT-PCR (data not shown). The same transcript is detected at later stages in the presomitic mesoderm and early skeletal muscle (Figure 1c). To confirm the restricted localization of Xα_1S mRNA, we examined its expression in mesodermal explants dissected at stage 10 (Figure 1b). DM but not VM explants expressed Xα_1S mRNA, and some minor labeling was observed in the lateral marginal zone (Figure 1c; 100% of expression in DMZ, 43% in LMZ, 0% in VMZ).

Since α_1S encodes L-type, dihydropyridine (DHP)-sensitive calcium channels, we sought to determine the presence of DHP binding sites in tissues obtained from stage 10 embryos. Explants were incubated with the DHP fluorescent analog 4,4-difluoro-7-styryl-4-bora-3α,4α-diaza-3-(s-indacene)-propionic acid (STBodipy-DHP) [3]; examination by confocal microscopy revealed specific label of the probe to DMZ but not to VMZ cells (Figure 1d). Labeling was also observed in animal caps, which could reflect the weak expression of Xα_1S mRNA detected by ISH or RT-PCR. It should be emphasized that these procedures were not intended to be quantitative, and thus, no correlation between the levels of VSCC mRNA and the protein expression or DHP staining can be derived from these observations. To determine if the presence of the messenger and DHP binding sites associates with elevated intracellular calcium levels, we evaluated the fluorescence arising from explants loaded with the intracellular calcium indicator Fluo-3. DMZ cells displayed higher steady levels of Fluo-3, suggesting high levels of free intracellular calcium as compared to VMZ and ecto-

Figure 1



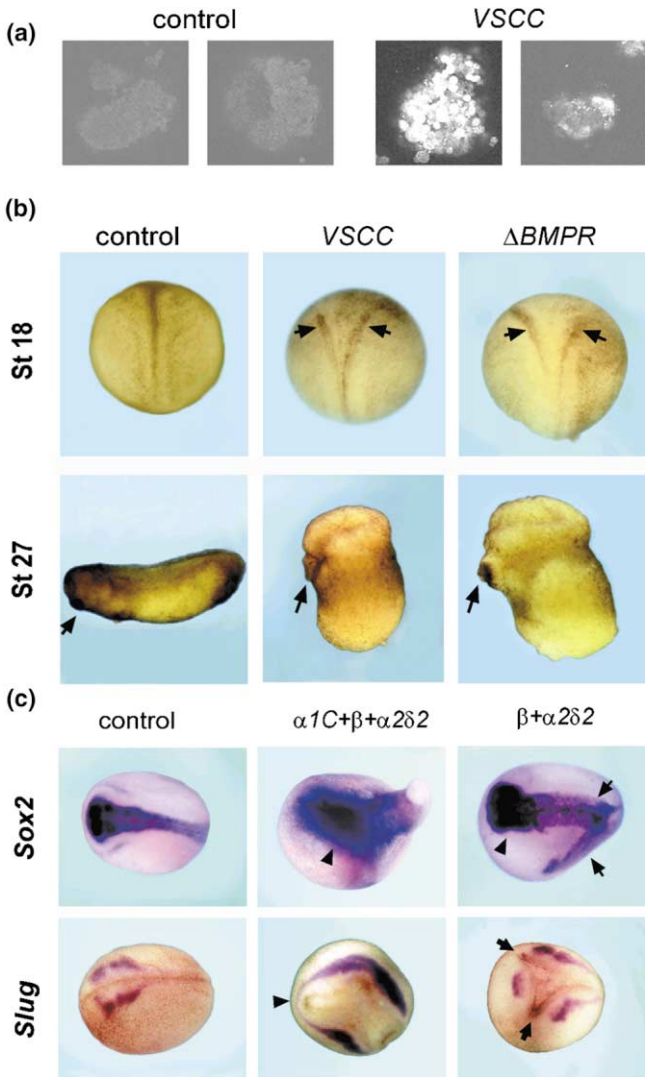
Expression patterns of $X\alpha_1S$ in the *Xenopus laevis* embryo. **(a)** A clone containing a 2 kb insert with an 89% homology to human α_1S was isolated. The GenBank accession number for $X\alpha_1S$ is AF421880. Nested RT-PCR experiments were conducted by using RNA isolated from whole embryos. RT-PCR analysis of the expression of $X\alpha_1S$ and $X\beta_3$ in oocytes (OO); embryos at blastula (St. 7), gastrula (St. 10), neurula (St. 18), and early tadpole (St. 35) stages; RT−, no reverse transcription. EF1α primers were used as controls. It should be noticed that this RT-PCR is not quantitative. **(b)** Schematic representation of dissection procedures. **(c)** Upper panels: whole-mount ISH analysis of the expression of $X\alpha_1S$ [22]. Left to right: stage 10 embryo, vegetal view showing the localization of the transcript in the dorsal mesoderm, above the blastopore lip; $X\alpha_1S$ is present in presomitic mesoderm (ps) and somites (s) at stages 18 and 26 (dorsal views, anterior to the left); the inset shows somitic localization of $X\alpha_1S$ in a tissue section. Lower panels: $X\alpha_1S$ ISH in explants dissected at stage 10 and cultured until the equivalent of stage 18; $X\alpha_1S$ mRNA is present in DMZ but not VMZ; some labeling is appreciated in lateral mesoderm (LMZ). **(d)** Upper panels: DMZ explants and AC exhibit specific DHP binding. Left to right, control DMZ explant preincubated with 20 μM nitrendipine; DMZ, VMZ, and AC preincubated for 5 min with 250 nMST of Bodipy-DHP (Molecular Probes). Insets show a transmitted light image of the corresponding explants. Lower panels: DMZ explants and AC display higher Ca^{2+} concentration than VM. Left to right: control, unloaded DM explant; explants of DMZ, VMZ, and AC incubated for 60 min with 10 μM of Fluo-3 (Molecular Probes); experiments representative of three similar measurements (four explants each).

dermal explants (Figure 1d). These results support a recent observation where levels of calcium were found to be higher in DMZ [6]. Similarly in the zebrafish, a dynamic pattern of elevation of the Ca^{2+} concentration is observed throughout the early developmental stages, although VSCC have not been explicitly implicated in its generation [17]. Taken together, our results demonstrate a restricted expression of functional L-type VSCC encoded by $X\alpha_1S$ in the DM during gastrulation.

VSCC and dorsalization

We injected the RNA-encoding L-type calcium channels transcribed from cloned cDNAs encoding heterologous α_{1C}, β_{1b}, and $\alpha_2\delta_2$ subunits into embryos at one-, two-, or four-cell stages. α_1 subunits conform the main ion-conducting subunit of VSCC, whereas β_{1b} and $\alpha_2\delta_2$ subunits do not directly participate in the passage of ions but contribute to membrane expression and modulate the function of α subunits. These transcripts were demonstrated to induce the expression of functional calcium channels when injected into *Xenopus* oocytes by voltage clamp (data not shown) and to increase the intracellular calcium concentration in tissues dissected from injected embryos as compared to uninjected controls (Figure 2a). At the equivalent of stage 18, one-cell stage animal pole-injected embryos displayed a dorsalized phenotype characterized by the appearance of a double axis (n = 120, 26%; Figure 2b) and enlargement of the dorsoanterior structures. At later stages (the equivalent of stage 27 of control embryos), the injected embryos showed gross morphological perturbations consistent with an expansion of dorsoanterior structures and a reduction of posterior development (n = 125, 98%). These morphological perturbations are similar to the dorsalized phenotype that results from the overexpression at the one-cell stage of the dorsalizing agents noggin and chordin or the blockade of the BMP-4 signal transduction cascade by expression of the dominant-negative BMP type I receptor (ΔBMP-R, Figure 2b). The double axis was also recognized by the expression of molecular markers such as *Sox-2*, a marker for neural plate cells [9], and *Slug*, a gene expressed in neural crest cells ([10]; Figure 2c). These results indicate that overexpression of VSCC leads to an exaggerated dorsalization of the embryo. We also overexpressed the β_{1b} and $\alpha_2\delta_2$ subunits and found a phenotype similar to that resulting from the coinjection of these two plus the α_{1C} RNA (Figure 2c). Expression of the accessory subunits of VSCC β_{1b} and $\alpha_2\delta_2$ also dorsalized the embryo. Control injections of equivalent amounts of an RNA encoding β-galactosidase or of an RNA encoding a calcium-acti-

Figure 2



Injection of heterologous VSCC RNA dorsalizes the embryo. **(a)** Injection of RNA encoding VSCC (α_{1C}, β_{1b}, and $\alpha_2\delta_2$ subunits) results in increased intracellular calcium levels in embryonic tissues. Representative confocal fluorescence images of Fluo-3-loaded DMZ explants from uninjected embryos (control) and injected embryos from the same batch and processed simultaneously are shown. **(b)** Embryos were injected at one-cell stage with the RNAs indicated in each frame (see text for details), and the morphology was evaluated at stages 18 (upper panels) and 27 (lower panels). Arrows indicate the double axis at stage 18 and the cement gland at stage 27. **(c)** Injection of 3 ng of VSCC RNA associates to expansion of the territory of expression of *Sox-2* and *Slug*, evaluated by ISH. Arrowheads indicate expansion of the territory of expression of the marker genes; arrows show double axis.

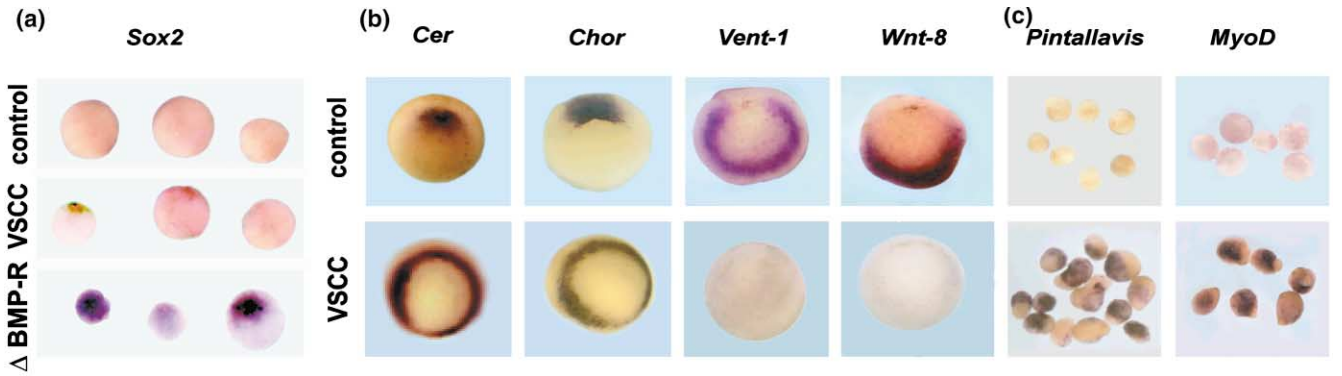
vated potassium channel did not result in morphological or endogenous gene expression alterations (data not shown). In other systems, these subunits do not participate in ion conduction, but they profoundly influence the amount of the α subunit that reaches the plasma membrane and forms functional channels [19, 20]. These results are compatible with the view that the excess of exogenous accessory subunit enhances the traffic of α to the cell surface. Misexpression of VSCC induces a phenotype similar to the one that results from overexpression of noggin or the inhibition of ΔBMP signaling. Thus, the elevation of the Ca^{2+} concentration could inhibit a signal transduction pathway distal to the binding of the BMPs to their receptor. We were able to rescue the effect of VSCC mRNA injection on the expression of chordin by coinjection of BMP-4 mRNA (data not shown). It has been shown that expression of a constitutively active form of calcineurin, a calcium-dependent protein phosphatase, results in a dorsalized phenotype, and a modulation of the phosphorylation of the BMP receptor has been proposed to underlay this effect [21]. Here we show that calcium channels mediate mesodermal patterning acting probably downstream of BMPs signals.

We tested the hypothesis that overexpression of VSCC directly leads to neural specification, as suggested by recent reports [2–5], and we studied the expression of *Sox-2* in ectodermal explants (animal caps) dissected at stage 10 from embryos previously injected at the one-cell stage with the RNAs encoding VSCC. These explants failed to express the neural marker, although animal caps taken from embryos injected with ΔBMPR showed a strong *Xsox-2* expression (Figure 3a). This contrasts with the proposal that activation of VSCC in the amphibian ectoderm directly leads to neural induction. It is possible that the levels of calcium entry obtained during our experiments were not sufficient to attain this effect or that the increase in the calcium level required to neuralize the ectoderm is not dependent on VSCC. An alternative explanation for the effect of VSCC misexpression is the expansion of neural inducer tissue or dorsal mesoderm. We therefore explored the effects of the misexpression of VSCC on the dorsoventral patterning of the mesoderm by evaluating the expression of several mesodermal markers. We observed a ventral expansion in the expression of dorsal mesoderm genes such as *Cerberus* and *Chordin* [7] and an inhibition in the expression of ventral mesoderm genes such as *Vent-1* [11] and *Wnt-8* [12] (Figure 3b). Additionally, explants of VMZ dissected at stage 10 from embryos injected with VSCC RNAs and cultured until the equivalent of stage 18 exhibited a strong expression of the dorsal mesoderm markers *Pintallavis* [13] and *MyoD* [14] (Figure 3c) and an elongated phenotype similar to the shape displayed by an explant of DMZ as consequence of convergent extension. These results indicate that misexpression of VSCC is able to induce dorsal characteristics to ventral mesoderm.

Blockade of VSCC inhibits the dorsalization of the mesoderm

To inhibit VSCC activity, we used peptidic toxins that specifically block L-type calcium channels. Exposing

Figure 3



Injection of heterologous VSCC RNA dorsalizes the mesoderm. **(a)** Animal caps taken from control embryos or from embryos injected with VSCC (α_{1C}, β_{1b}, and $\alpha_2\delta_2$ subunits) or ΔBMP-R RNA and analyzed for *Sox-2* expression. No expression of *Sox-2* can be detected. Arrows indicate β-galactosidase-activity staining. **(b)** Vegetal view of control embryos (upper panels) and embryos injected at one-cell stage with VSCC mRNA (lower panels) evaluated by ISH of *Cerberus* (*Cer*), *Chordin* (*Chor*), *Vent-1*, and *Wnt-8*. Notice the ectopic expression of *Cer* and *Chor* induced by VSCC injection and the inhibition in the expression of *Vent-1* and *Wnt-8*. **(c)** VMZ taken at stage 10 from control embryos (upper panels) and embryos injected with VSCC mRNA (lower panels), cultured until the equivalent of stage 18; the expression of *Pintallavis* or *MyoD* was analyzed. Notice that VSCC RNA injection induces the expression of these markers in the VMZ.

whole embryos at stage 6 to calcicludine [15] or taicatoxin [16] resulted in blocking gastrulation at stage 10, suggesting an effect on mesodermal development. This effect was partially reverted after extensive washing of the embryos in toxin-free solution. DMZ explants dissected at stage 10 and cultured until the equivalent of stage 18 undergo characteristic extension and express dorsal markers such as MyoD (Figure 4a), in contrast with VMZ explants that remain round and do not express these markers (Figure 4d). Exposure of DMZ explants to calcicludine significantly inhibited convergent extension and the appearance of MyoD (Figure 4b; n = 38, 85% of inhibition). This effect of calcicludine on convergent extension and MyoD expression was reversed by treatment under the same conditions with the toxin but with DMZ dissected from embryos previously injected with chordin mRNA, showing that the calcicludine treatment was not toxic for the mesodermal explants (Figure 4c; n = 32, 23% of inhibition).

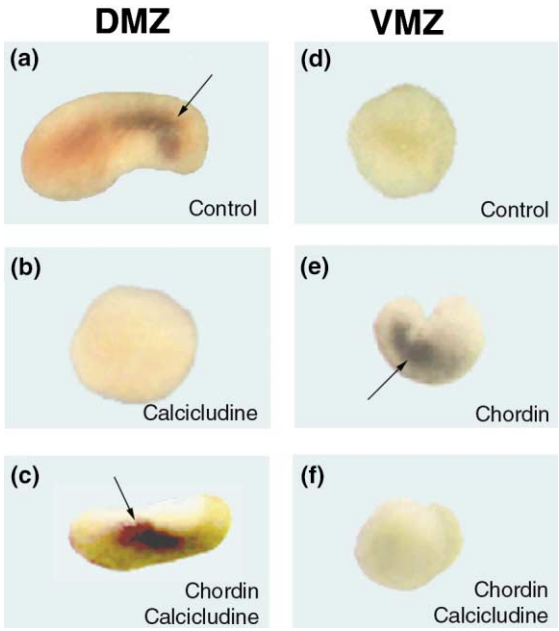
VMZ explants dissected from embryos previously injected with chordin RNA showed a dorsalized phenotype characterized by its extension and MyoD expression (Figure 4e) that could be blocked by treatment with calcicludine (Figure 4f; n = 36, 90% of inhibition). The sensitivity of VMZ dissected from chordin-injected embryos to calcicludine is consistent with an induction of VSCC expression. Thus, overexpression of VSCC leads to an expansion of dorsal territories, whereas their blockade impairs the acquisition of differentiated features in dorsal cells. It is interesting to notice that recent studies show that Ca^{2+}/calmodulin and Ca^{2+}/inositol 1,4,5-trisphosphate (IP_3) promote ventral cell fates [23–25]. A possible explanation for this discrepancy in the role of calcium as a dorsalizing or ventralizing agent could lie in the subcellular source for the cytosolic elevation of the calcium concentration. Thus, it has been well demonstrated in other systems that even different pathways of calcium entry through the plasma membrane can differentially affect gene expression [26]. Accordingly, calcium-sensitive proteins involved in dorsalizing or ventralizing pathways may display distinct subcellular distributions.

Gastrulation involves a complex set of morphogenetic movements. The main engine producing the driving force for gastrulation is thought to be convergent extension resulting from mediolateral intercalation of deep cells in the DMZ [18]. Our results show that blockage of VSCC inhibits convergent extension. However, the absence of dorsal mesoderm markers in the treated explants suggests that the inhibition of convergent extension is a consequence of the prior misspecification of the dorsal mesoderm. In addition, the expression of VSCC in the ventral mesoderm is enough to dorsalize the ventral cells in terms of cell movement and gene expression. Our findings thus suggest that calcium is a key component of early mesodermal patterning.

Acknowledgements

We thank A. Ruiz i Altaba, M. Bonner-Fraser, and C. Stern for helpful comments on a previous version of the manuscript. We are grateful to R. Grainger, K.W.Y. Cho, R.T. Moon, M. Sargent, and J.B. Gurdon for cDNA probes and F. Espinoza for technical assistance. This investigation was supported by grants from Fondo Nacional de Ciencia y tecnología-Chile (FONDECYT) (1961084, 1990570, and 2980019), Millennium Nucleus in Developmental Biology (P99-137-F), the Human Frontier Science Program (RG0042/98), Fundación G. Puelma, and Universidad de Chile.

Figure 4



Blockade of endogenous VSCC inhibits dorsalization of the mesoderm. **(a)** Control explant of DMZ dissected at stage 10 and cultured until the equivalent of stage 18 shows characteristic convergent extension and expression of *MyoD* revealed by ISH (indicated by arrow). **(b)** Treatment with calcicludine (10 μM) inhibits convergent extension and *MyoD* expression. **(c)** Treatment with calcicludine (10 μM) is not able to inhibit convergent extension and *MyoD* expression in DMZ taken from embryos injected with chordin mRNA. Arrows show *MyoD* expression. **(d)** Control explants from VMZ do not display extension nor *MyoD* expression. **(e)** VMZ taken at stage 10 from embryos injected with 1 ng of chordin mRNA at the one-cell stage; notice the elongation of the explants and *MyoD* expression (arrow). **(f)** VMZ explants treated with 10 μM calcicludine; notice the inhibition of the extension and *MyoD* expression. In each experiment above, 30 explants were analyzed.

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