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Short Contribution

Expression of L-type Ca^{2+} channel during early embryogenesis in *Xenopus laevis*

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ABSTRACT The mechanisms involved in the first step of neurogenesis, i.e. neural induction, are poorly understood, particularly in terms of the signalling pathway. In a recent work it has been shown that in urodeles the activation of L-type calcium channels is sufficient to trigger neural induction. In order to substantiate a possible role of this channel in early development in anurans, we have detailed the kinetics of the expression and the localization of the α_1 subunit of L-type calcium channel in the early stages of *Xenopus laevis* embryogenesis using immunological techniques. We observed that the expression of the α_1 subunit started during blastulation, where a cytoplasmic labeling was observed. At the onset of gastrulation α_1 was targeted to the plasma membrane of the dorsal and the ventral ectoderm. Some labeling was found in the mesoderm but never in the endoderm. This expression seems to be general, since similar results have been obtained in anurans *(Xenopus)* and in urodeles *(Pleurodeles)*. In addition, we found that the α_o subunit of the G_o protein is expressed simultaneously and strictly colocalized with the α_1 subunit of the L-type calcium channel. The role of this channel and its regulation by G_o protein during early neurogenesis is discussed.

KEY WORDS: *Ca^{2+} channel, early embryogenesis, neurogenesis, Xenopus laevis*

The L-type calcium channel is an oligomeric protein, with one main subunit, α_1, that serves as both pore and voltage sensor. It is expressed in a wide variety of cells and mediates voltage-controlled calcium entry into cells (Hofmann *et al.*, 1994). L-type calcium channels have been shown to play a key role in excitation-contraction coupling in skeletal muscle (Rios and Brum, 1987) and to fulfil a pivotal function in coupling synaptic stimulation to regulation of gene expression thus contributing to neuronal plasticity (Murphy *et al.*, 1991).

Voltage-operated calcium channels also appear to be developmentally significant for the control of neuronal differentiation (review in Spitzer, 1991, 1994). Furthermore, increasing evidence indicates that L-type calcium channels are also involved in the commitment of embryonic cells towards the neural pathways. Neural induction, which is the initial step in neural development, occurs during gastrulation and results from an interaction between the inductive dorsal mesoderm and the responsive ectoderm which will give rise to neural structures (for review see Saxén, 1989; Gilbert and Saxén, 1993). The molecular mechanism triggering neural induction is still unclear. However, it is now well established that inductive molecules interact with structures located at the plasma membrane (Born *et al.*, 1986) and that transducing signals connect this binding to nuclear events, i.e. neural-specific gene expression (Kintner, 1992). In *Pleurodeles waltl* embryos functional L-type calcium channels are transitorily present on ectoderm cell membranes from pregastrula up to the late gastrula stages (Leclerc *et al.*, 1995). Furthermore, it has been demonstrated that L-type calcium channels are directly implicated in the transduction of the neuralizing signal brought about by Concanavalin A (ConA) (Moreau *et al.*, 1994). ConA is a lectin which has been shown to have neural inductive properties in the urodele *Triturus pyrrhogaster* (Takata *et al.*, 1981) and in *Pleurodeles waltl* (Gualandris *et al.*, 1985). Taken together, these data strongly argue for a determinant role of the L-type calcium channel in the triggering of neural induction in urodeles.

In the anuran amphibian *Xenopus laevis* embryos it has also been shown that ConA binds to competent ectoderm cells and is able to induce neural structures (Grunz, 1984; Tacke and Grunz, 1986).

Therefore, we wanted to determine whether L-type calcium channels also play an important role during neural induction in anurans. In a first approach to this question, we studied the temporal expression of the L-type calcium channels and its location in *Xenopus laevis* embryos. For this purpose, we used a mouse

Abbreviations used in this paper: ConA, Concanavalin A; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; $G\alpha_0$, alpha subunit of G_0 protein.; MAB, monoclonal antibody; NAM, normal amphibian medium; TRITC, tetramethylrhodamine isothiocyanate.

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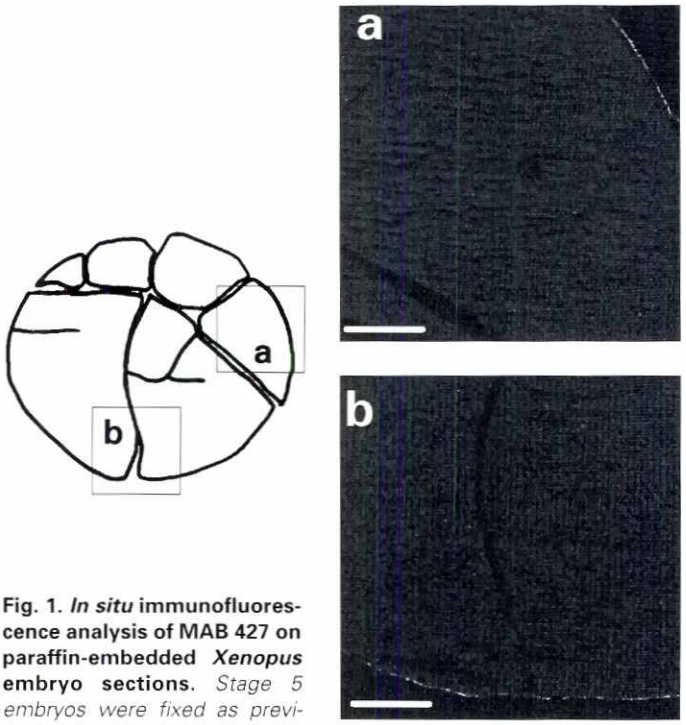

Fig. 1. *In situ* immunofluorescence analysis of MAB 427 on paraffin-embedded *Xenopus* embryo sections. *Stage 5 embryos were fixed as previously described (Levi et al., 1987) and immunohistochemistry was carried out on 10 μm serial sections. No labeling was observed in the animal* **(a)** *or the vegetal* **(b)** *half of the embryo. Bar, 50 μm.*

anti-dihydropyridine binding complex monoclonal immunoglobulin (MAB 427) directed against the α_1 subunit of the L-type calcium channel extracted from transverse tubules of rabbit skeletal muscle (Morton *et al.*, 1988). We showed that the L-type calcium channel began to be expressed before gastrulation in *Xenopus laevis* embryo, and therefore may be involved in the transduction mechanism of the neuralizing signal in anurans.

MAB 427 is a commercial antibody, and its reactivity was tested using immunoblotting techniques on protein-enriched fractions from rat and *Xenopus* adult skeletal muscle; a major single band was found at the same molecular weight in rat and in *Xenopus* and MAB 427 recognized transversal structures on the adult skeletal muscle of *Xenopus* (data not shown). In rat, Morton *et al.* (1988) have clearly demonstrated that MAB 427 recognizes the α_1 subunit of the skeletal L-type calcium channel. According to these properties, we concluded that MAB 427, had an immuno-like reactivity against α_1 in *Xenopus*.

The expression of the α_1 subunit of the L-type calcium channel was first investigated on sections of the whole embryo from the 16-cell stage up to the beginning of gastrulation. At stage 5 (16-cell embryos), no labeling was found other than that due to non-specific trapping of antibodies by the vitelline membrane (Fig. 1a,b). We can assess that this labeling is not specific since similar staining is obtained when the secondary antibody was applied alone.

At stage 7 (early blastula stage), the embryo is composed of the presumptive ectoderm, consisting of small cells, and the presumptive endoderm, constituted by large rounded cells. At the site of contact between ectoderm and endoderm, namely the marginal zone, mesodermal induction is taking place (for review see Woodland, 1989; Slack, 1994). The α_1 labeling was observed in the whole presumptive ectoderm (Fig. 2a) and in small cells of the whole marginal zone (Fig. 2b,c), whereas endoderm was unlabeled (Fig. 2d). In the area between ectoderm and endoderm, we noted some bigger positively stained cells, which were probably induced mesodermal cells (Fig. 2b,c).

At stage $10^{1/4}$, the morphogenetic movements of gastrulation begin and appearance of the blastopore lip allows the dor-

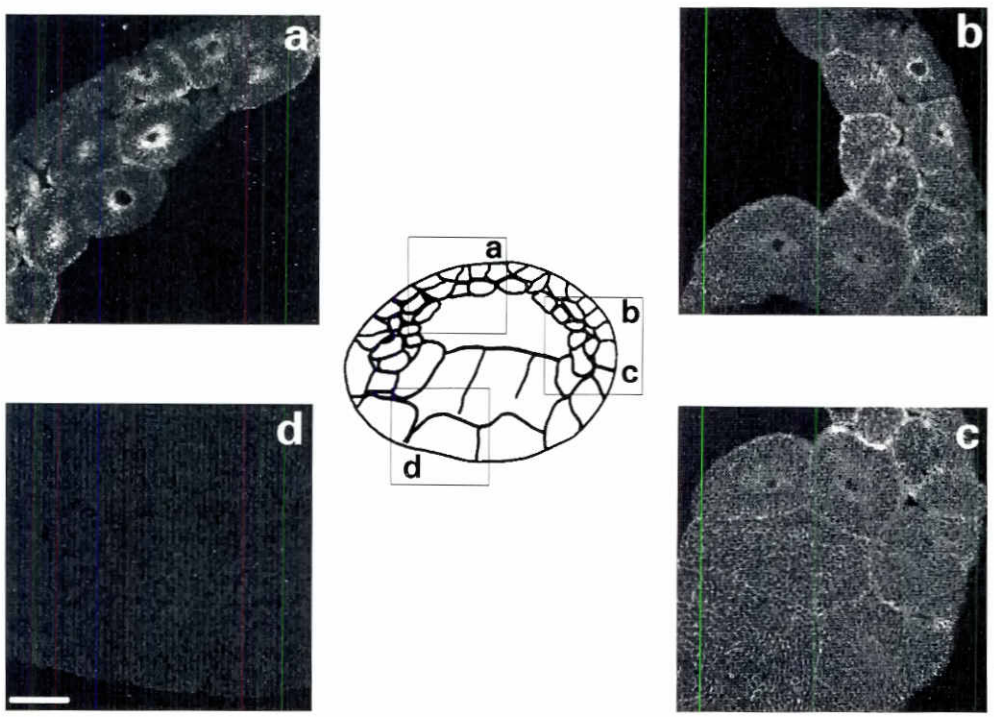

Fig. 2. Immunohistochemistry on serial sections of a stage 7 embryo. *In stage 7 embryos (treated as for Fig. 1), mesectoderm is positive, whereas endoderm is not labeled.* **(a)** *Ectoderm;* **(b,c)** *marginal zone;* **(d)** *endoderm. Bar, 50 μm.*

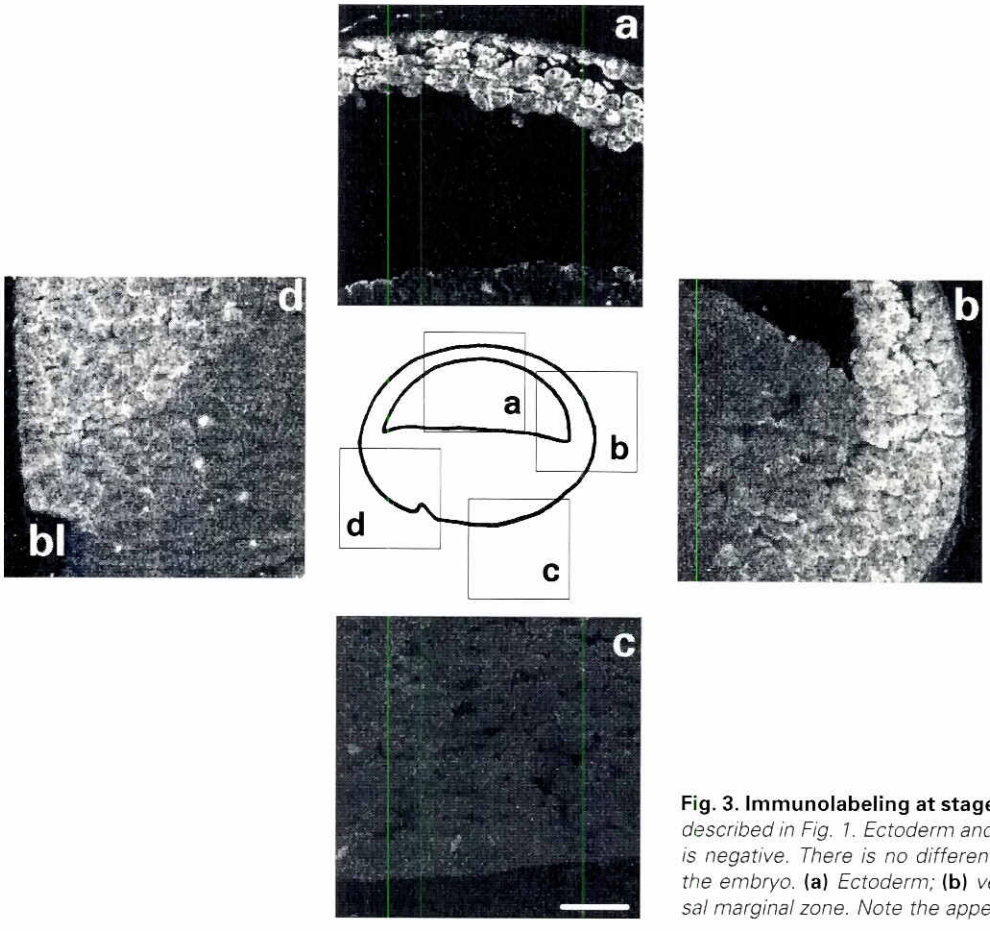

Fig. 3. Immunolabeling at stage $10^{1/4}$. *Stage-$10^{1/4}$ embryos were treated as described in Fig. 1. Ectoderm and mesoderm are labeled, whereas endoderm is negative. There is no difference between the dorsal and ventral sides of the embryo.* **(a)** *Ectoderm;* **(b)** *ventral marginal zone;* **(c)** *endoderm;* **(d)** *dorsal marginal zone. Note the appearance of the blastopore lip (bl). Bar, 50 μm.*

sal and ventral sides of the embryo to be distinguished. The ectoderm is composed of two cell layers. Whereas the mesoderm is localized at the marginal zone and begins its invagination, the endoderm still occupies the vegetal half of the embryo.

Like at stage 7, the endoderm of stage $10^{1/4}$ embryos remained unlabeled (Fig. 3c). On the other hand, immunoreactivity was observed throughout the ectoderm (Fig. 3a) and at the marginal zone (Fig. 3b,d). At the marginal zone, we observed a clear boundary between positive cells and negative endodermal ones. At the dorsal marginal zone, positive cells were localized above the blastopore lip, the region corresponding to the invaginating marginal zone as described in the fate map (Dale and Slack, 1987). It should be underlined that there was no difference in labeling between dorsal and ventral sides of the marginal zone (Fig. 3b vs d).

In addition, as clearly seen on sections of stage 7 embryos (Fig. 2), the protein was expressed both at the plasma membrane and around the nucleus, the nucleus itself being negative. In order to study more precisely the distribution of the α_1 subunit of L-type calcium channel at the cellular level, additional observations were performed on isolated cells obtained after dissociation of the presumptive ectoderm dissected from stage 7, stage 8 and stage $10^{1/4}$ embryos. Confocal microscopy analysis of isolated cells allowed us to clearly visualize the cytoplasmic labeling (Fig. 4).

When embryos were dissected at stage 7 (mid-blastula stage, i.e. before the beginning of zygotic gene expression), the cytoplasm was heterogeneously labeled. Different positive domains deep in the cytoplasm were observed. A faint immunoreactivity was also found at the plasma membrane level (Fig. 4a).

When the dissection was carried out at stage 8 (late-blastula stage, i.e., onset of midblastula transition, Gerhart, 1980; Newport and Kirschner, 1982a,b), a strong labeling was seen to be localized at the level of the plasma membrane and in the cytoplasm beneath (Fig. 4b). We noted that the labeling was localized in patches in the cell and at the plasma membrane.

At stage $10^{1/4}$ (early gastrula), the ectoderm is composed of two cell layers which possess some difference in neural competence (Grunz, 1984). We therefore decided to study them independently. The two sheets of ectodermal cells can be easily separated and the cells dissociated when placed in Ca^{2+}/Mg^{2+}-free medium. We did not observe any difference in the location or in the intensity of the labeling of cells from these two layers (Fig. 4c,d). The immunoreactivity was seen, like at stage 8, at the plasma membrane and in the cytoplasm just beneath. Only part of the cell was labeled, suggesting an eventual capping of the channels triggered by the dissociation, as previously described by Gualandris *et al.* (1983).

The transducing GTP-binding protein Go has been described to regulate L-type calcium channel activity (Sweeney and Dolphin, 1992). In addition, a previous study indicates that the α

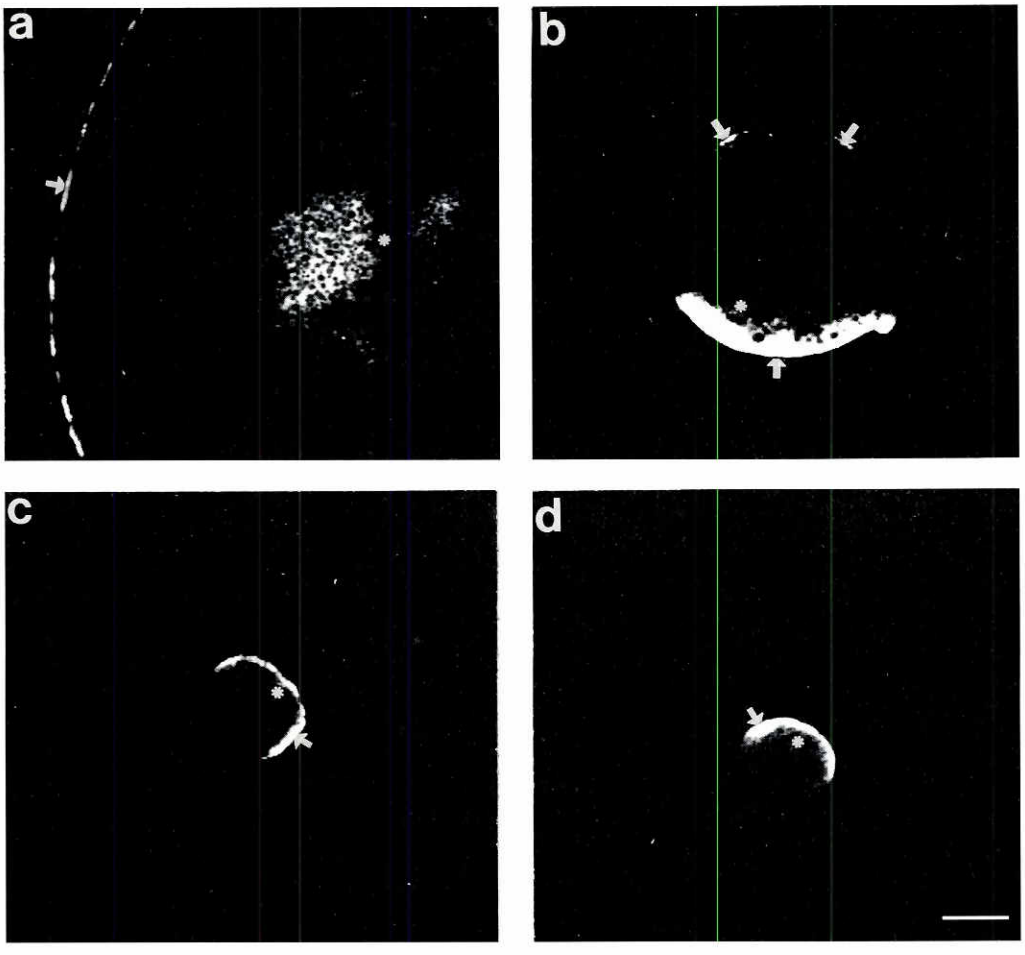

Fig. 4. Immunocytochemistry on dissociated cells. *Ectoderms were dissected at the precise stages indicated and incubated in a Ca^{2+}/Mg^{2+}-free medium before fixing. Immunodetection was carried out as described.* **(a)** *Dissection was carried out at stage 7. Only part of a cell is shown. The apparently very large diameter observed is due to compression between two coverslips during the mounting procedure. Labeling is seen deep in the cytoplasm (asterisk) and at the plasma membrane (arrow);* **(b)** *ectoderm dissected at stage 8. No labeling remains deep in the cytoplasm. α_1 is at the plasma membrane (arrows) and in the cytoplasm near it (asterisk);* **(c)** *stage $10^{1/4}$ (external layer of ectoderm);* **(d)** *stage $10^{1/4}$ (internal layer of ectoderm). At this stage, for both cell layers, labeling is at the plasma membrane (arrow) and in the cytoplasm just beneath (asterisk). Bar, 50 μm for a; 25 μm for b,c and d.*

subunit of the G_o protein ($G\alpha_o$) is expressed in the ectoderm cells of *Pleurodeles waltl* embryos and that its expression is correlated with the ability of the ectoderm cells to respond to neural inducing signal (called neural competence) (Pituello *et al.*, 1991). Consequently, it was important to study the respective localizations of the α_1 subunit of the L-type calcium channel and the α_o subunit of the G_o protein. Double immunolabeling was thus carried out on isolated ectoderm cells and visualized under epifluorescence microscopy (Fig. 5).

At each stage tested (7, 8 and 10), we found that 80% of the α_1-positive cells were also α_0 positive. Furthermore, the pattern of α_1 and α_0 immunoreactivity was found identical, restricted to patches on the plasma membrane and strictly colocalized (Fig. 5). However, in a few cells α_1 immunostaining was not found colocalized with α_0 one (Fig. 5b and c).

The data obtained both on sections and with isolated cells indicate that in *Xenopus laevis* while at early blastula (stage 7) the labeling for α_1 is mostly observed in the cytoplasm of the presumptive ectoderm cells, from midblastula (stage 8) the α_1 subunit of the L-type calcium channel is present both in the plasma membrane of ectodermal and mesodermal cells and in the cytoplasm just beneath the membrane. These observations suggest that the α_1 subunit of the L-type calcium channel is targeted to cell membranes in a progressive manner from early blastula through to the beginning of gastrulation and is therefore expressed at the right time and place during early development to be a good candidate for a role in the triggering of neural induction. Several observations further emphasize the above hypothesis. (i) Both dorsal and ventral sides of stage 10 ectoderm display immunoreactivity for α_1. Indeed, graft experiments of dorsal mesoderm onto ventral ectoderm of *Xenopus* gastrula indicate that ventral ectodermal cells have the ability to respond to neural stimuli (Gimlich and Cooke, 1983; Smith and Slack, 1983). (ii) The immunostaining pattern that we observed for $G\alpha_0$ in *Xenopus* ectoderm is in agreement with that found by Pituello *et al.* (1991) at equivalent stages in *Pleurodeles* embryos. This suggests that like in *Pleurodeles* , in *Xenopus* the acquisition of the state of neural competence by the ectoderm is under the control of the expression of the L-type calcium channel in the plasma membrane and/or the Go protein which has certainly a determinant role in the L-type calcium channel regulation.

Experimental Procedures

Embryos

Eggs, obtained by injecting female *Xenopus laevis* with 750 U.I. human chorionic gonadotrophin 15 h before laying, were fertilized with dilacerated testis *in vitro*. Embryos were dejellied with 2% cysteine hydrochloride

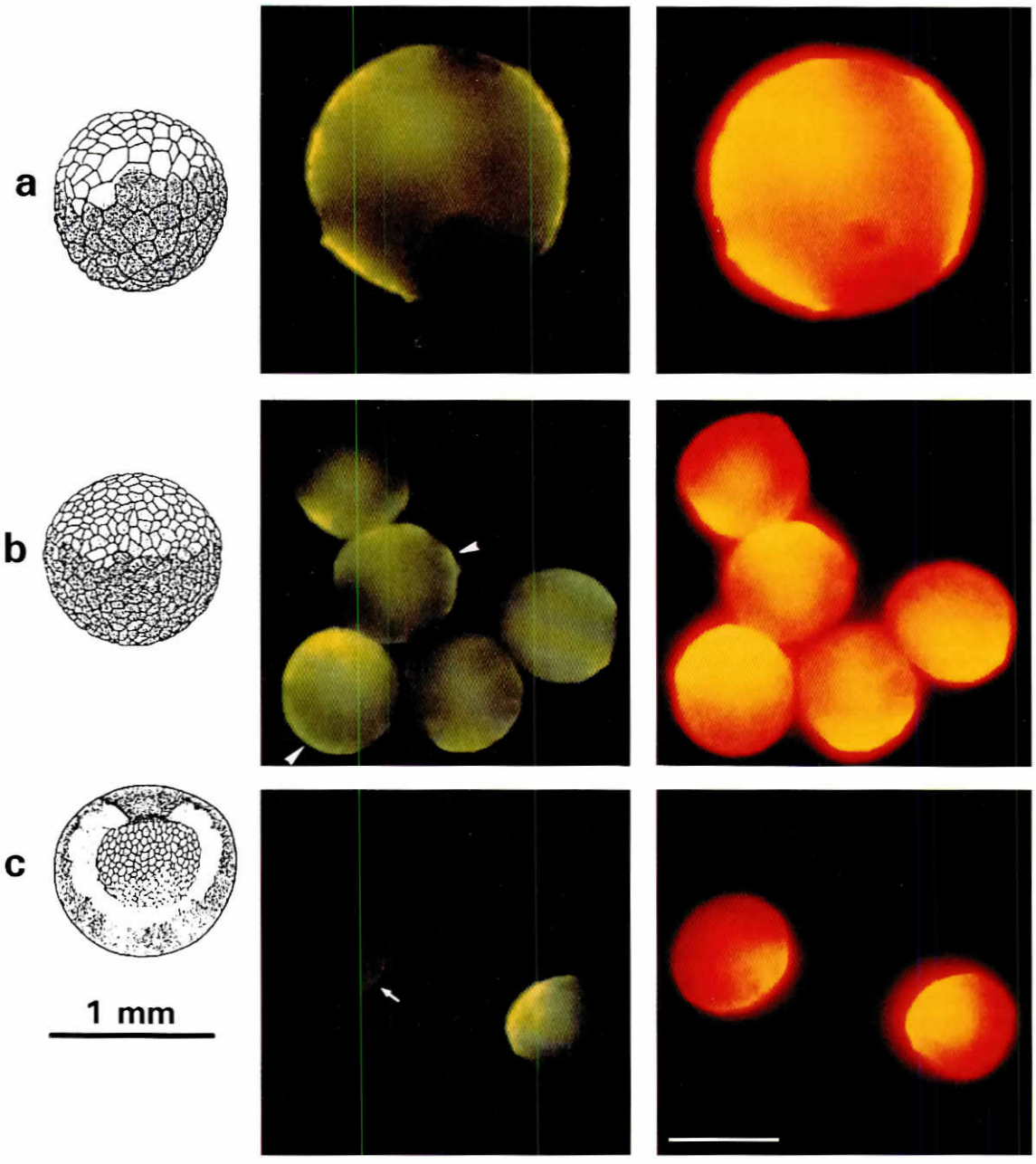

Fig. 5. Colocalization of α_1 subunit of L-type Ca^{2+} channel and α subunit of Go protein. *Animal cap of stage 7* **(a)**, *stage 8* **(b)** *and stage 10* **(c)** *were dissected and cells dissociated in a Ca2+/Mg2+ free medium before fixing. Double labeling on the same cell was carried out as described in Experimental procedures. Left panel: α_1 immunolabeling; right panel: $G\alpha_0$ immunolabeling. Note that the two different antibodies labeled the same region of the plasma membrane. However some slight differences may exit in the colocalization. Arrowhead in* **(b)** *points to α_1 positive and $G\alpha_0$ negative domains. Conversely arrow in* **(c)** *points to α_1 negative and $G\alpha_0$ positive domains. Bar, 35 μm.*

and raised in 0.1X NAM (Normal Amphibian Medium: 0.1X is 11 mM NaCl, 0.2 mM KCl, 0.1 mM $NaHCO_3$, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.01 mM Na_2 EDTA, 0.16 mM Na_2HPO_4, 0.04 mM NaH_2PO_4, pH 7.4) at 22°C. Embryos were staged according to Nieuwkoop and Faber (1967).

Embryos of different stages were dissected in 1X NAM. Tissues were dissociated in Ca^{2+}/Mg^{2+} free Barth's medium (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 2 mM Na_2HPO_4, 0.1 mM KH_2PO_4, 0.5 mM EDTA, pH 8.5) as previously described (Barth and Barth, 1959) before cells were fixed in 3.5% formaldehyde in 1X NAM.

Alternatively, embryos of different stages were fixed as previously described (Levi *et al.*, 1987). Briefly, embryos were rapidly immersed in isopentane (-80°C) and transferred into methanol at -80°C for 60 h. They were then incubated successively in methanol at -20°C, +4°C and at room temperature for 2 h each. After 2 baths of 15 min in toluene, they were paraffin embedded and sectioned (10 mm).

Immunological techniques

Primary antibodies

Commercial mouse anti-dihydropyridine binding complex monoclonal immunoglobulins (MAB 427, Chemicon, Temecula, USA): this antibody is directed against the α_1 subunit of L-type Ca^{2+} channel extracted from transverse tubules of rabbit skeletal muscle. It is described to cross-react with rat, human, and murine L-type Ca^{2+} channels (Morton *et al.*, 1988). It was used at 1/100 dilution for immunocytochemistry and 1/30 for immunohistochemistry. Polyclonal immunoglobulins directed against the G-protein subunit $G\alpha_0$ (Go, a gift of Dr. Homburger) was used at 1/30 dilution (Pituello *et al.*, 1991).

Secondary antibodies

Fluorescein (FITC)-conjugated goat anti-mouse immunoglobulins

(Nordic, France) were used at 1/100 dilution. Rhodamine (TRITC)-conjugated goat anti-rabbit immunoglobulins (Immunotech) were used at 1/50 dilution. Goat anti-mouse-biotinylated immunoglobulins (Amersham) were used at 1/50 dilution. FITC-conjugated streptavidin (Amersham) was used at 1/50 dilution.

Immunolabeling

Dissociated cells were incubated with the primary antibodies for 30 min. After washing as described below, they were incubated with the appropriate secondary antibodies for 30 min. After each incubation step, 3-5 min washes were necessary to eliminate unfixed antibodies. After each washing step, the cells were gently centrifuged for 2 min (5 g). After washing, coverslips were mounted in Mowiol 4-88 and viewed using an epifluorescence confocal microscope (Zeiss), illuminated by an Argon laser. For double-labeled samples, the observations were carried out using a Leitz Dialux epifluorescence microscope equipped with rhodamine and fluorescein filter sets; photographs were taken on color Ektachrome 160 film (160 ASA). Sections were immunolabeled as described for dissociated cells except that they were incubated for 60 min with the different antibodies. Moreover, the secondary antibody used was biotinylated. The presence of antigen-antibody complex was detected after incubation with streptavidin-FITC for 15 min.

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References

BARTH, L.G. and BARTH, L.J. (1959). Differentiation of cells of the *Rana pipiens* gastrula in unconditioned medium. *J. Embryol. Exp. Morphol. 7:* 210-222

BORN, J., HOPPE, P., JANECZEK, J., TIEDEMANN, H., and TIEDEMANN, H. (1986). Covalent coupling of neuralizing factors from *Xenopus* to Sepharose beads: no decrease of inducing activity. *Cell Differ. 19:* 97-101.

DALE, L., and SLACK, J.M.W. (1987). Regional specification within the mesoderm of early embryos of *Xenopus laevis. Development 99:* 279-295.

GERHART, J.G. (1980). Mechanisms regulating pattern formation in the amphibian egg and early embryo. In *Biological Regulation and Development, 2* (Ed. R.F. Goldeberger). Plenum Press, New York, pp. 133-315.

GILBERT, S.F., and SAXÉN, L. (1993). Spemann's organizer: models and molecules. *Mech. Dev. 41:* 73-89.

GIMLICH, R.L. and COOKE, J. (1983). Cell lineage and the induction of the second nervous system in amphibian development. *Nature 306:* 471-473.

GRUNZ, H. (1984). Early embryonic induction: the ectodermal target cells. In *The Role of Cell Interactions in Early Neurogenesis. Series A, Life Sci. Ed. Vol. 77* (Eds. A-M. Duprat, A.C. Kato and M. Weber). Plenum Press, New York, pp. 21-38.

GUALANDRIS, L., ROUGE, P. and DUPRAT, A-M. (1983) Membrane changes in neural target cells studied with fluorescent lectin probes. *J. Embryol. Exp. Morphol. 77:* 183-200

GUALANDRIS, L., ROUGE, P. and DUPRAT, A-M. (1985). Target cell surfaces glycoconjugates and neural induction in an amphibian. *J. Embryol. Exp. Morphol. 86:* 39-51.

HOFMANN, F., BIEL, M., and FLOCKERZI, V. (1994). Molecular basis for Ca^{2+} channel diversity. *Annu. Rev. Neurosci. 17:* 399-418.

KINTNER, C. (1992) Molecular bases of early neural development in *Xenopus* embryos. *Annu. Rev. Neurosci. 15:* 251-284.

LECLERC, C., DUPRAT, A-M. and MOREAU, M. (1995). *In vivo* labelling of L-type calcium channels by fluorescent dihydropyridine: correlation between ontogenesis of the channel and the acquisition of neural competence in ectoderm cells from *Pleurodeles waltl* embryos. *Cell Calcium 17 :* 216-224.

LEVI, G., CROSSIN, K.L. and EDELMAN, G.M. (1987). Expression sequences and distribution of two primary cell adhesion molecules during embryonic development of *Xenopus laevis. J. Cell Biol. 105:* 2359-2372.

MOREAU, M, LECLERC, C., GUALANDRIS-PARISOT, L., and DUPRAT, A-M. (1994). Elevation of internal calcium mediates neural induction in the amphibian embryo. *Proc. Natl. Acad. Sci. USA 91:* 12639-12643.

MORTON, M.E., CAFFREY, J.M., BROWN, A.M. and FROEHNER, S.C. (1988). Monoclonal antibody to the α_1-subunit of the dihydropyridine-binding complex inhibits calcium currents in BC3H1 myocytes. *J. Biol. Chem. 263:* 613-616.

MURPHY, T.H., WORLEY, P.F. and BARBARAN, J.M. (1991). L-type voltage-sensitive calcium channels mediate synaptic activation of immediate early gene. *Neuron 7:* 625-635.

NEWPORT, J. and KIRSCHNER, M. (1982a). A major developmental transition in early *Xenopus* embryos: I Characterization and timing of cellular changes at the midblastula stage. *Cell 30:* 675-686.

NEWPORT, J. and KIRSCHNER, M. (1982b). A major developmental transition in early Xenopus embryos: II Control of the onset of transcription. *Cell 30:* 687-696.

NIEUWKOOP, P.D. and FABER, J. (1967). *Normal Table of Xenopus laevis (Daudin): A Systematic and Chronological Survey of the Development of the Fertilized Egg till the End of Metamorphosis,* 2nd ed. North Holland, Amsterdam.

PITUELLO, F., HOMBURGER, V., SAINT-JEANNET, J-P., AUDIGIER, Y., BOCKAERT, J. and DUPRAT, A-M. (1991). Expression of the guanine nucleotide-binding protein G_o correlates with the state of neural competence in the amphibian embryo. *Dev. Biol. 145:* 311-322.

RIOS, E. and BRUM, G. (1987). Involvement of dihydropyridine receptors in excitation contraction coupling in skeletal muscle. *Nature 325:* 717-720.

SAXÉN, L. (1989). Neural induction. *Int. J. Dev. Biol. 33:* 21-48.

SLACK, J.M.W. (1994). Inducing factors in *Xenopus* early embryos. *Curr. Biol. 4:* 116-126.

SMITH, J.C. and SLACK, J.M.W. (1983) Dorsalization and neural induction: properties of the organizer in *Xenopus laevis. J. Embryol. Exp. Morphol. 78:* 299-317.

SPITZER, N.C. (1991). A developmental Handshake: neuronal control of ionic currents. *J. Neurobiol. 22:* 659-673.

SPITZER, N.C. (1994). Spontaneous Ca^{2+} spikes and waves in embryonic neurons: signaling systems for differentiation. *Trends Neurosci. 17:* 115-118.

SWEENEY, M.T. and DOLPHIN, A.C. (1992). 1,4-dihydropyridines modulate GTP hydrolysis by G_o in neuronal membranes. *FEBS Lett. 310:* 66-70.

TACKE, L. and GRUNZ, H. (1986). Electron microscopy study of the binding of Con A-gold to superficial and inner ectoderm layers of *Xenopus laevis* and its relation to the neural-inducing activity of this lectin. *Roux Arch. Dev. Biol. 195:* 158-167.

TAKATA, K, YAMAMOTO, K.T. and OZAWA, R. (1981). Use of lectins for analyzing embryonic inductions. *Roux Arch. Dev. Biol. 190:* 92-96.

WOODLAND, H.R. (1989). Mesoderm formation in *Xenopus. Cell 59:* 767-770.

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