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Spatiotemporal Aspects of Prolactin-Induced Calcium Signaling

Thomas Ducret,[1] Bruno Sorin,[1] Anne Marie Vacher,[1] and Pierre Vacher[1,2]

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We have examined the spatiotemporal aspects of PRL-induced Ca^{2+} signals using high-speed fluo-3 confocal imaging. We found that PRL stimulated Ca^{2+} entry and intracellular Ca^{2+} mobilization. Ca^{2+} influx was seen as a peripheral increase in $[Ca^{2+}]i$ without amplification in the nucleus region. Intracellular Ca^{2+} mobilization was seen as a propagating intracellular calcium wave with a strong amplification in the nuclear region. The amplitude of PRL-induced Ca^{2+} increases would be sufficient to stimulate cell proliferation. Furthermore, PRL induced an increase in $[^3H]$thymidine incorporation. These data suggest that PRL would be able to induce mitogenesis through a Ca^{2+}-dependent pathway.

KEY WORDS: Signal transduction; calcium imaging; cell proliferation; high-speed confocal fluorescence imaging; prolactin receptor.

INTRODUCTION

PRL is a polypeptide hormone that is mainly synthesized in lactotrophic cells of the anterior pituitary of vertebrates. One of the best-characterized roles of PRL is its ability to stimulate cell proliferation. For example, the growth of normal rat glial cells is, at least in part, controlled by PRL [1]. Several studies have also documented the involvement of PRL in the growth control of various tumors. However, the molecular events between PRL binding to the cell surface receptors and the expression of biological response are not well understood. To study early events in PRL signal transduction, we have developed a CHO (Chinese hamster ovary) cell line (CHOTSE32) stably transfected with the cDNA of the long form of rabbit mammary PRL-R. These CHO-transfected cells respond to PRL by stimulating the cotransfected milk protein gene promoter, proving that such cells are fully capable of transmitting the PRL signal and that PRL-R is functional. Using this cell line, we demonstrated that exposure of cells to physiological concentrations of PRL resulted in an increase in the cytosolic free calcium concentration ($[Ca^{2+}]i$) [2] by stimulating both Ca^{2+} entry and mobilization from intracellular Ca^{2+} stores.

Calcium ions are a potent intracellular messenger involved in several physiological responses (cell proliferation, apoptosis, . . .); we have compared the spatiotemporal aspects of PRL-induced Ca^{2+} signals in the CHOTSE32 cell line and in a cell line naturally expressing the long form of the PRL receptor, the U87-MG cell line. For this purpose we have used a high-speed (video rate) fluo-3 confocal imaging.

MATERIAL AND METHODS

Cell Culture

Human glioblastoma astrocytoma (U87-MG) cells were obtained from the European Collection of Cell Cultures. Cells were cultured in DMEM (Dulbecco-Modified

[1] Laboratoire de physiologie et physiopathologie de la signalisation cellulaire, UMR CNRS 5543, université de Bordeaux2, 146, rue Léo Saignat, 33076 Bordeaux Cédex, France.

[2] To whom correspondence should be addressed. Tel: (33) 5 57 57 16 68. E-mail: Pierre. Vacher@umr5543.u-bordeaux2.fr

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Earl's Medium) containing 10% (v/v) fetal calf serum (FCS). CHO cells (TSE32) were obtained from Dr. J. Djiane (INRA, Jouy en Josas, France). They were grown in Ham's F12 medium containing 10% (v/v) FCS. Both cell models were maintained at 37°C in a humidified atmosphere gassed with 95% air, 5% CO_2.

Calcium Imaging

Cells were loaded with fluo-3 AM. $[Ca^{2+}]i$ imaging was performed using a laser-scanning Insight system (Meridian, Okemos, USA). The system generates images of faintly fluorescent objects at high speed (25 images/s), and every frame has a very good signal-to-noise ratio. Images may be observed directly, as a continuous movie, through the microscope eyepieces and captured with a low-light-level video-camera (Darkstar 800, Photonic Science). Excitation of fluo-3 fluorescence was provided by the 488-nm line of a 100-m*W* argon laser and fluorescence emission was long pass-filtered (515 nm). The confocal output aperture slit was set to a minimum opening compatible with a good signal-to-noise ratio/frame (in most cases, optical slice width was 1–2 μ*M*). Images were acquired at video rate, recorded on a video-cassette recorder (U-matic), and viewed, in real-time, on a video monitor.

The recorder video frames were digitized at a resolution of 512 × 512 × 8 bits using an image processing system (Synetic, Noesis, Orsay, France) and analyzed using Visilog 4 software (Noesis, Orsay, France) on an Athlon 600 PC. Each frame was corrected for camera dark current and background fluorescence by subtracting a frame obtained at the end of each experiment. $[Ca^{2+}]i$ variations were expressed, on a pixel-by-pixel basis, as ratios of fluorescent images F(t)/F(t0) (normalized images), where *F*(*t*) refers to the fluo-3 fluorescent image at a given time(*t*) and *F*(*t0*) to the fluorescent image at resting level. Dividing *F*(*t*) images by *F*(*t0*) image corrects for differences in shading and fluo-3 concentration. Routinely, images were spatially filtered by averaging a matrix of 3 × 3 pixels to improve the signal-to-noise ratio. The resulting ratio images were represented in pseudo 3-dimensional pictures (with the F(t)/F(t0) ratio on a *z*-axis perpendicular to the cell plane) and in color levels from blue to red (pseudo-colors) to improve visual observation of the relative changes in $[Ca^{2+}]i$, from low to high, respectively. Time course plots for successive video image ratios were generated by averaging the ratios of all pixels within the image of a given cell, or within areas of interest. Cell nucleus position was determined by acridine orange treatment. Calcium imaging experiments were carried out on cells bathed in a standard extracellular solution containing (in m*M*): 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 0.3 Na_2HPO_4, 0.4 KH_2PO_4, 4 $NaHCO_3$, 5 glucose, 10 HEPES. The osmolality of this solution was adjusted to 300–310 mosmol/kg with sucrose, and pH was adjusted to 7.3 ± 0.01 with NaOH.

RESULTS

Intracellular Localization of the PRL-Induced Calcium Response

We investigated spatiotemporal aspects of PRL-induced Ca^{2+} signals using high-speed fluo-3 confocal imaging of single cell $[Ca^{2+}]i$ dynamics. We found different Ca^{2+} increase localizations in response to ovine PRL in CHO cells. This heterogeneity of cellular responses was observed even in the same field of cells stimulated simultaneously. Representative examples of two types of response are shown in Fig. 1. Sequences of false-color three-dimensional images chosen at the indicated time illustrate the spatiotemporal $[Ca^{2+}]i$ variations induced by 4 n*M* oPRL (Fig. 1). The first type of response, shown in Fig. 1A, is a "peripheral increase" in $[Ca^{2+}]i$. At early time points after the beginning of the response, the increase in $[Ca^{2+}]i$ was restricted to the subplasmalemmal region (Fig. 1Ac). oPRL caused a homogenously distributed rise around the periphery of the cell. The time course of this rise in $[Ca^{2+}]i$ was slow (Fig. 1Ab). Ca^{2+} ions slowly propagated in a decremental manner from the plasma membrane to the cytosol, toward the cell center. Note that, in the case of a peripheral increase, the $[Ca^{2+}]i$ increase was greater in the subplasmalemmal region than in the nucleus. This may be due to fast calcium buffering near the membrane.

The second type of response was named "calcium wave". A rapid, local rise in $[Ca^{2+}]i$ (Fig. 1Bb) was triggered at, usually, only one locus in the cell (Fig. 1Bc). The rise in $[Ca^{2+}]i$ rarely initiated at two separate points. Starting from the initial locus, the $[Ca^{2+}]i$ increase showed a propagating wave, which spread into the cytosol along the long axis of the cell, invaded the nucleus, and reached the opposite extremity (Fig. 1Bc). The time course of the $[Ca^{2+}]i$ wave is illustrated in Fig. 1Bd. The delay in $[Ca^{2+}]i$ increase in successive areas along the CHO cell illustrates the advance of the wave front, which propagated in a non-decremental manner. The decline of the high $[Ca^{2+}]i$ levels took several minutes, and no polarity in the $[Ca^{2+}]i$ distribution was apparent in the descending phase of the peak (data not shown). The nuclei showed intranuclear $[Ca^{2+}]i$ variations (black squares) slightly in excess vs. cytosolic $[Ca^{2+}]i$ increases (grey triangles and

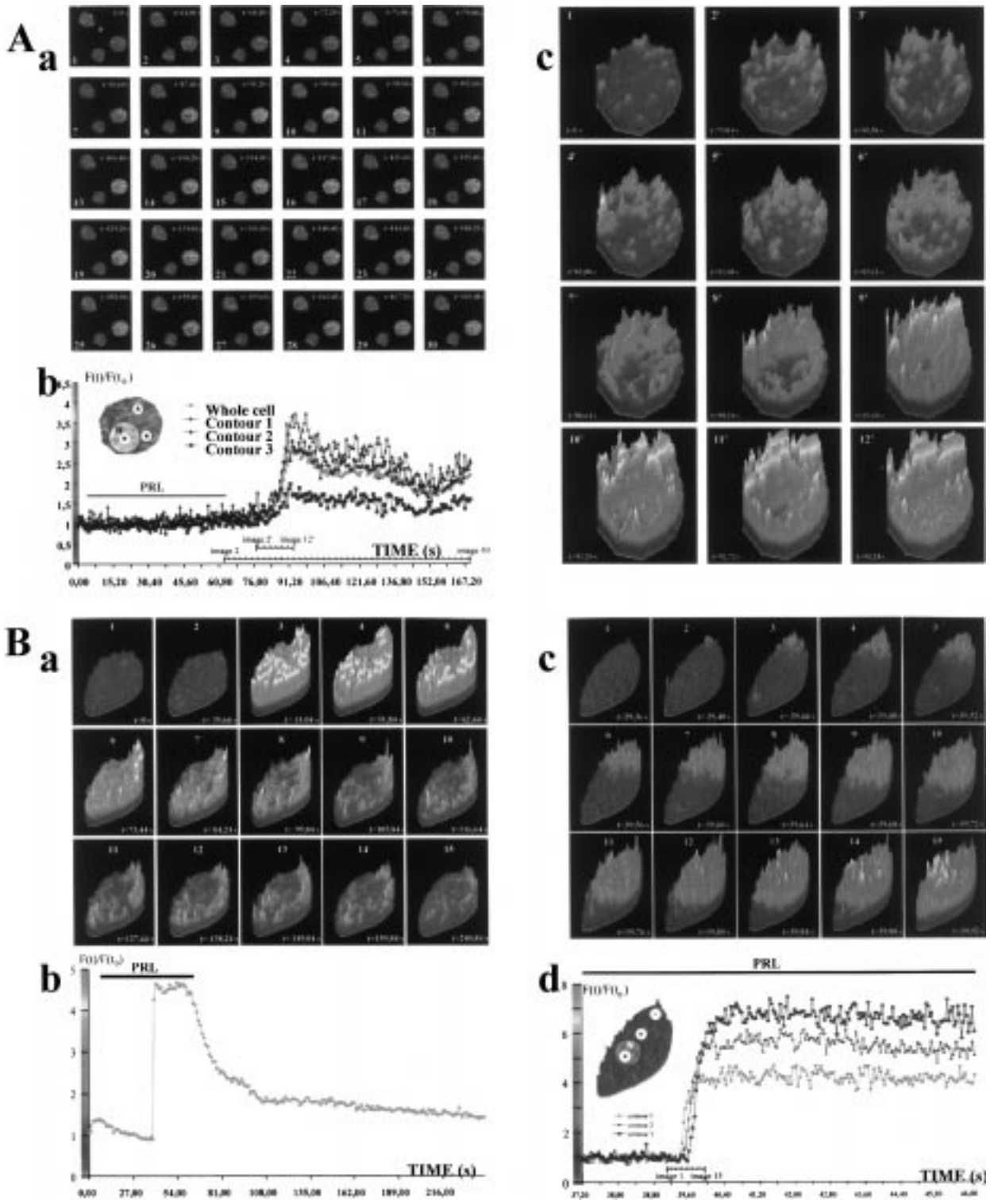

Fig. 1. Spatiotemporal aspects of PRL-induced Ca^{2+} increase in CHO cells. PRL induces a slow, maintained Ca^{2+} influx (A), or a fast, transient intracellular Ca^{2+} mobilization (B). Only the intracellular Ca^{2+} mobilizations are associated to an increase in the Ca^{2+} concentration in the nuclear region.

red circles), as shown in Fig. 1Bd. Note that the fluorescence was, at rest, equivalent in the nucleus and cytosol.

The use of 0 mM Ca^{2+}-containing extracellular medium showed that the first type of response (« peripheral increase ») corresponded to an influx of Ca^{2+} ions, whereas the second type (« calcium wave) correspond to a release of Ca^{2+} stored in intracellular compartment (data not shown). Like in U87 cells, (Fig. 2A), the fast ("calcium wave") response was more frequently observed at the lowest PRL concentrations ($<$ 4 n*M*), whereas the slow (« peripheral increase ») response was more frequently observed at higher concentrations (Fig. 2A).

The same PRL-induced Ca^{2+} responses (« calcium wave » and « peripheral increase ») were observed in U87 cells (data not shown), suggesting that these responses were not due to the transfection process.

PRL-Induced Proliferation of U87-MG Human Glioma Cell Line

PRL-induced influences were assessed using two methods: (1) [^{3}H] thymidine incorporation measurements and (2) colorimetric methylthiotetrazole assay in the U87 cells.

Figure 2 Ba illustrates the effects of human PRL (0.04 n*M*–40 n*M*) on DNA synthesis in U87-MG cells, as determined by [^{3}H]thymidine uptake. When cells were incubated with hPRL for 48 h in DMEM minus fetal calf serum (FCS), PRL induced a significant stimulation of proliferation from 0.4–40 n*M* ($p < 0.001$). hPRL concentrations of 0.4–4 n*M* gave an optimal response (about 100% increase in [^{3}H]thymidine uptake). Note that a 2-fold smaller increase in [^{3}H] thymidine uptake was obtained at a 10-fold higher concentration of PRL (40 n*M*).

Figure 2Bb illustrates the effects of hPRL (0.4–40 n*M*) and epidermal growth factor (EGF, 1.5–15 n*M*) on cell growth in U87-MG cells, as determined by MTT colorimetric assay. We also found a stimulation of cell proliferation. Note that 0.4–4 n*M* PRL concentrations were as efficient as 1.5–15 n*M* EGF on the proliferation of U87-MG cells. From these data it appears that PRL concentrations inducing intracellular Ca^{2+} mobilization (associated to intranuclearCa^{2+} increases) are the concentrations efficient on cell proliferation.

DISCUSSION

Digital image analysis was used to resolve the temporal and spatial variations in cytoplasmic Ca^{2+} concentrations ([Ca^{2+}]i) induced by PRL.

The first type of Ca^{2+} dynamic is a peripheral increase in [Ca^{2+}]i resulting from Ca^{2+} influx from the extracellular space, as it disappeared in Ca^{2+}-free medium. This PRL-induced Ca^{2+} increase resulting from calcium influx also propagated from the cell membrane to the cell interior with a decremental magnitude.

In the second type of PRL response, the PRL-induced intracellular Ca^{2+} mobilization is localized (initiated) in one discrete cytosolic area (or, more rarely, two areas) of the cell and propagated throughout the whole cell as a wave. These nondecremental calcium waves relied entirely on the release of Ca^{2+} from intracellular stores, because they could be generated even when the cells were incubated in Ca^{2+}-free medium. Several hypotheses for the mechanisms of Ca^{2+} waves have been proposed [3]. We have no convincing argument for any of them. Briefly, the Ca^{2+} wave propagation mechanism may implicate: (1) Ca^{2+}-sensitized inositol 1,4,5 trisphosphate-induced Ca^{2+} release; (2) Ca^{2+}-induced Ca^{2+} release; (3) Ca^{2+}-activation of phospholipase C coupling with inositol 1,4,5 trisphosphate-induced Ca^{2+} release, or (4) inositol 1,4,5 trisphosphate diffusion. The mechanisms may depend on the cell type, but are still controversial. There is limited understanding of the physiological significance of these propagating Ca^{2+} signals. For example, they are thought to establish polarity in eggs at fertilization, coordinate ciliary beating in airway epithelia, direct fluid secretion across epithelial cells, and regulate neurite extensions.

One intriguing observation from imaging experiments is that, in response to PRL, the nuclear Ca^{2+} was greater than the cytoplasmic Ca^{2+}, but only in the case of intracellular calcium release and not Ca^{2+} influx. Note that the basal fluorescence was similar in all parts of the cell and, thus, that under unstimulated conditions, there was no way of distinguishing the cell nucleus. Thus, nuclear Ca^{2+} appears to be regulated independently from cytoplasmic Ca^{2+}. These data suggest that production of the intracellular messenger (IP3 ?,. . .) responsible for PRL-induced intracellular Ca^{2+} mobilization may be required for [Ca^{2+}]i to increase in the nuclear region. Furthermore, immunocytochemical studies have revealed that the IP3 receptor is present in the perinuclear fraction of the endoplasmic reticulum and on the nuclear envelope [4]. In the same way, changes in either cytosolic or nucleoplasmic IP3 levels have been shown to control nuclear [Ca^{2+}] [5]. However, the observation that free [Ca^{2+}] within the cell nucleus appears to be higher than in the cytoplasm is an unexpected finding that would be explained if intranuclear [Ca^{2+}] were regulated by nuclear membrane-dependent processes, or may be artefactual and result from modified behavior of the dye in the

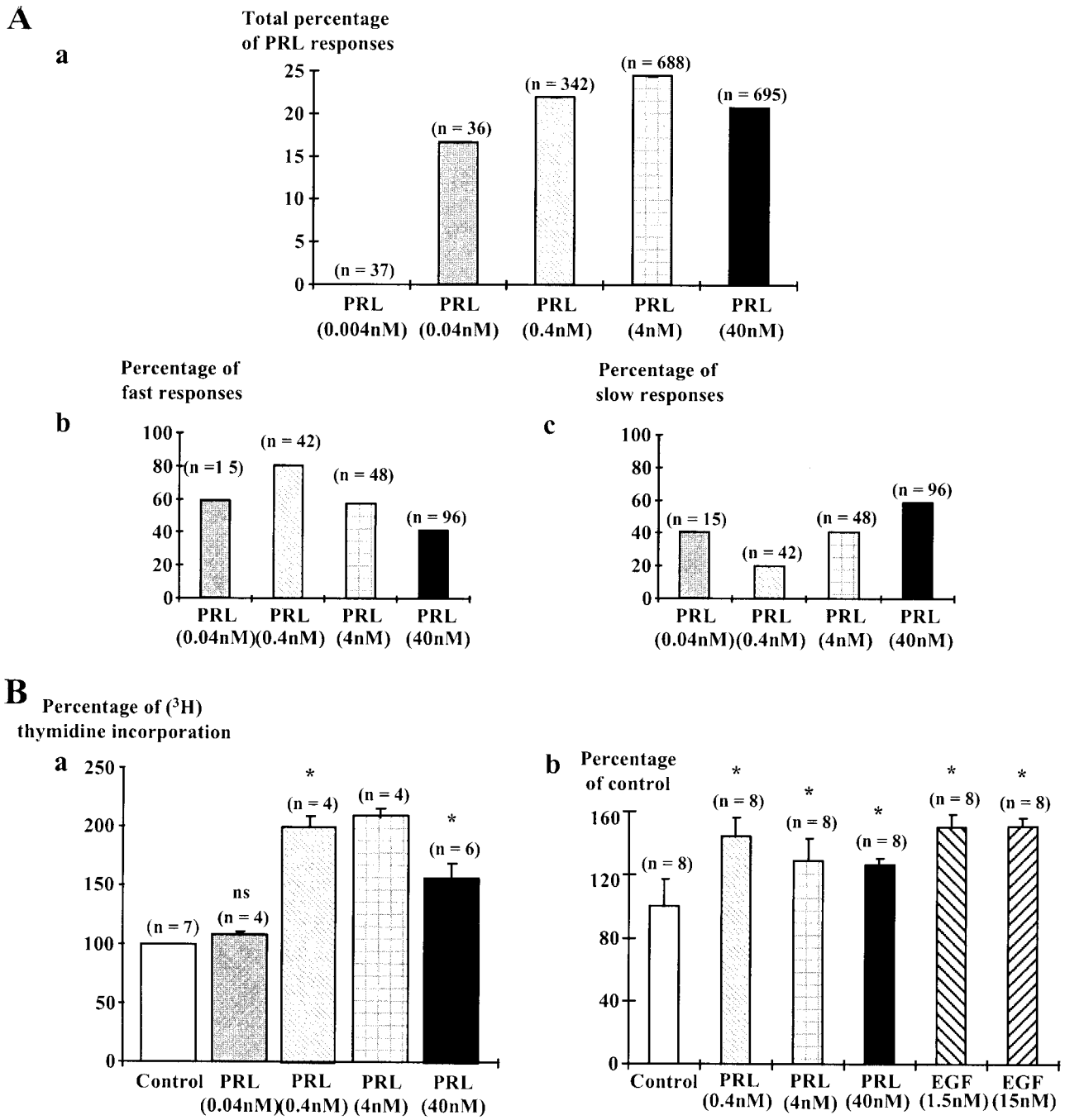

Fig. 2. Calcium responses (A) and cell proliferation (B) induced by PRL in U87-MG cells. A. *a*) Total percentage of PRL responses. *b*) and *c*) Percentage of fast (b) and slow responses (c) among responding cells. B. *a*) and *b*) U87-MG cell proliferation was determined by (^{3}H)thymidine incorporation (a) and MTT method (b). Asterisk indicates highly significant difference vs. control ($p < 0.01$). The values shown are the mean ± SEM.

nuclear environment, variability in autofluorescence of the various cell regions, or dye sequestration in organelles (for review see [6,7]. Thus, the topic of nuclear Ca^{2+} signaling is beset by discrepant observations of substantial nuclear cytoplasmic gradients. Ca^{2+} release and uptake were demonstrated in isolated rat liver nuclei (for review see [8]). More recently, the use of nucleoplasm-targeted aequorin has provided evidence for a nuclear

calcium barrier [9]. On the other hand, regarding the large pores that cross the nuclear membranes, many works (for review see [6]) claim that Ca^{2+} diffuses freely in and out of the nucleus and that nuclear [Ca^{2+}] passively reflects [Ca^{2+}]i. These authors attributed the nucleocytoplasmic gradient to artefacts linked to the methods used for Ca^{2+}-level determination (see above). Clearly, the many Ca^{2+}-signaling mechanisms that may impinge on nuclear Ca^{2+} will remain a topic of controversy and debate for some time (for review see [7]).

The present results also show that PRL has a dose-dependent stimulating effect on the proliferation of U87-MG cells. Two methods ([^{3}H]-thymidine incorporation and MTT assay) were used to measure cell proliferation. The concentration dependence of PRL-induced cell proliferation showed a bell-shaped profile, resembling the dose-dependent relationship of the percentage of cells responding to PRL with a [Ca^{2+}] increase. Note that PRL-induced cell proliferation was more pronounced at PRL concentrations that principally produced intracellular Ca^{2+} mobilization (0.4–4 n*M*) associated with nuclear Ca^{2+} increases. There is increasing evidence that membrane ion channels are involved in cell differentiation and cell-cycle control. Such a role of ion channels in PRL-induced cell proliferation in U-87 cells is currently under investigation in our lab.

In conclusion, the high-speed fluo-3 confocal imaging has allowed us to study the spatiotemporal aspects of the PRL-induced Ca^{2+} responses and to show that, in part of the cell population, PRL induced Ca^{2+} increases localized in the cell nucleus and fast Ca^{2+} waves. Further work is needed to characterize in greater detail the signal transduction pathways through which the PRL-R regulates physiological and pathophysiological cellular functions.

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