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Ca^{2+} is released from the nuclear tubular structure into nucleoplasm in C6 glioma cells after stimulation with phorbol ester

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Abstract It is well established that cellular Ca^{2+} is an important messenger that controls many nuclear functions but the source of nuclear Ca^{2+} is far from clear. It has long been thought that Ca^{2+} is translocated from the cytosol over a long distance to activate the nuclear transcription machinery. However, this model is at best an incomplete one. With the aid of confocal microscopy, we observed tubules extended deep inside the nucleus of C6 cells in agreement with previous studies (Fricker et al. (1997) J. Cell Biol. 136, 531–544). When cells were stimulated with phorbol 12-myristate 13-acetate or phorbol 12,13-diacetate, Ca^{2+} was released from these tubules. DiOC6(3), a vital marker for intracellular membranes, stained the tubule in the nucleus of the same cell used for Ca^{2+} imaging. Moreover, results from labelling the cells with rhodamine 123 further indicate that the tubule was formed by a double-membraned invagination with mitochondria inside. Studies with acridine orange showed that chromatin was excluded from the tubules. Taken together, our results demonstrate that the nuclear tubule is a structural entity responsible for the release of Ca^{2+} into the nucleoplasm after stimulation with phorbol ester.

Key words: Nuclear Ca^{2+}; Nuclear tubular structure; Invagination; Confocal microscopy

1. Introduction

Changes in the level of intracellular free Ca^{2+} regulate an array of cellular processes ranging from muscle contraction, gene expression and some forms of cell death [1–3]. It is now well established that Ca^{2+} signals in response to extracellular stimuli usually arise from the internal Ca^{2+} mobilization by the messenger inositol 1,4,5-trisphosphate (IP3) and the influx of Ca^{2+} through the membrane channels [4]. After stimulation, Ca^{2+} signals are terminated by the efflux of Ca^{2+} through the activation of Ca^{2+}-ATPases on the plasma membrane and by the re-uptake of Ca^{2+} into the endoplasmic reticulum (ER) [4]. In secretory cells, exocytosis is another mechanism to extrude Ca^{2+} to the extracellular environment [5]. Recently, a hierarchy of subcellular Ca^{2+} events that underlie the global Ca^{2+} signals has been demonstrated. The best known paradigm for such organization is indicated by the progressive recruitment of Ca^{2+} blips and puffs [6–8]. However, the regulation of Ca^{2+} inside the cell nucleus remains unclear [9–11].

In eukaryotic cells, the nucleus is the most prominent organelle which contains genetic materials. Most of the time, the cell nucleus is surrounded by the nuclear envelope (NE), a double-membraned structure with many nuclear pore complexes (NPC) which allow the diffusion of molecules below 50 kDa across the cyto-nucleoplasmic interface [12,13]. Since Ca^{2+} is a small ion, the diffusion of Ca^{2+} through the NPC was then accepted as a mechanism for the synchronous Ca^{2+} signals in the cytosol and cell nucleus upon stimulation [14]. However, data from patch-clamp studies demonstrate that the NPC are not freely permeable to ions [15]. Furthermore, it has been demonstrated that the nucleus has the ability to govern its own Ca^{2+} signals independently. For example, a number of Ca^{2+} transporters and enzymes for the phosphoinositide pathway have been identified in the outer and inner nuclear membrane [16,17].

In the present study, confocal microscopy of C6 cells was used to investigate the source of Ca^{2+} in the nucleus. Our results indicate that (i) tubular structures were found inside the nucleus of C6 cells; (ii) these tubular structures were derived from the invagination of the nuclear envelope; (iii) these structures were surrounded by chromatin; and (iv) after activation with phorbol ester, Ca^{2+} can be released into the nucleoplasm from these structures.

2. Materials and methods

2.1. Materials

Fluo-3/AM, DiOC6(3), acridine orange and rhodamine 123 were purchased from Molecular Probes. Phorbol 12,13-diacetate, phorbol 12-myristate 13-acetate and other chemicals were from Sigma.

2.2. Cell culture

C6 cells (from American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco). The cells were passaged twice weekly. For imaging studies, cells were seeded on coverslips in 35-mm diameter culture dishes at a cell density of 10^4/ml and incubated at 37°C, under an atmosphere of 5% CO_2. Subconfluent monolayer cells were used for experiments at day 3 after passage.

2.3. Determination of the intracellular Ca^{2+} level and the nuclear invagination by confocal laser scanning microscopy

Fluo-3, a Ca^{2+}-sensitive fluorescent indicator compatible with laser excitation, was used to monitor changes in intracellular Ca^{2+}. C6 cells grown on a coverslip were loaded with fluo-3/AM (2 μg/ml) at room temperature for 30 min to 1 h in Na^+-*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) buffer (concentration in mM: 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES, final pH 7.2). After washing, cells were incubated in HEPES buffer and changes in intracellular Ca^{2+} were measured using a confocal imaging system (Molecular Dynamics, USA) with Nikon objective (Plan-Apo, ×60, n.a. 1.4). For fluorescence determinations, excitation and emission were 488 nm and above 510 nm, respectively. Fluorescence images were collected at room temperature.

After Ca^{2+} experiments, the voltage of the photomultiplier was decreased until no fluorescent image was obtained, then DiOC6(3) (final concentration 0.01 μM) was added to determine the morphology of the ER/NE. In some experiments, cells were labelled with rhod-

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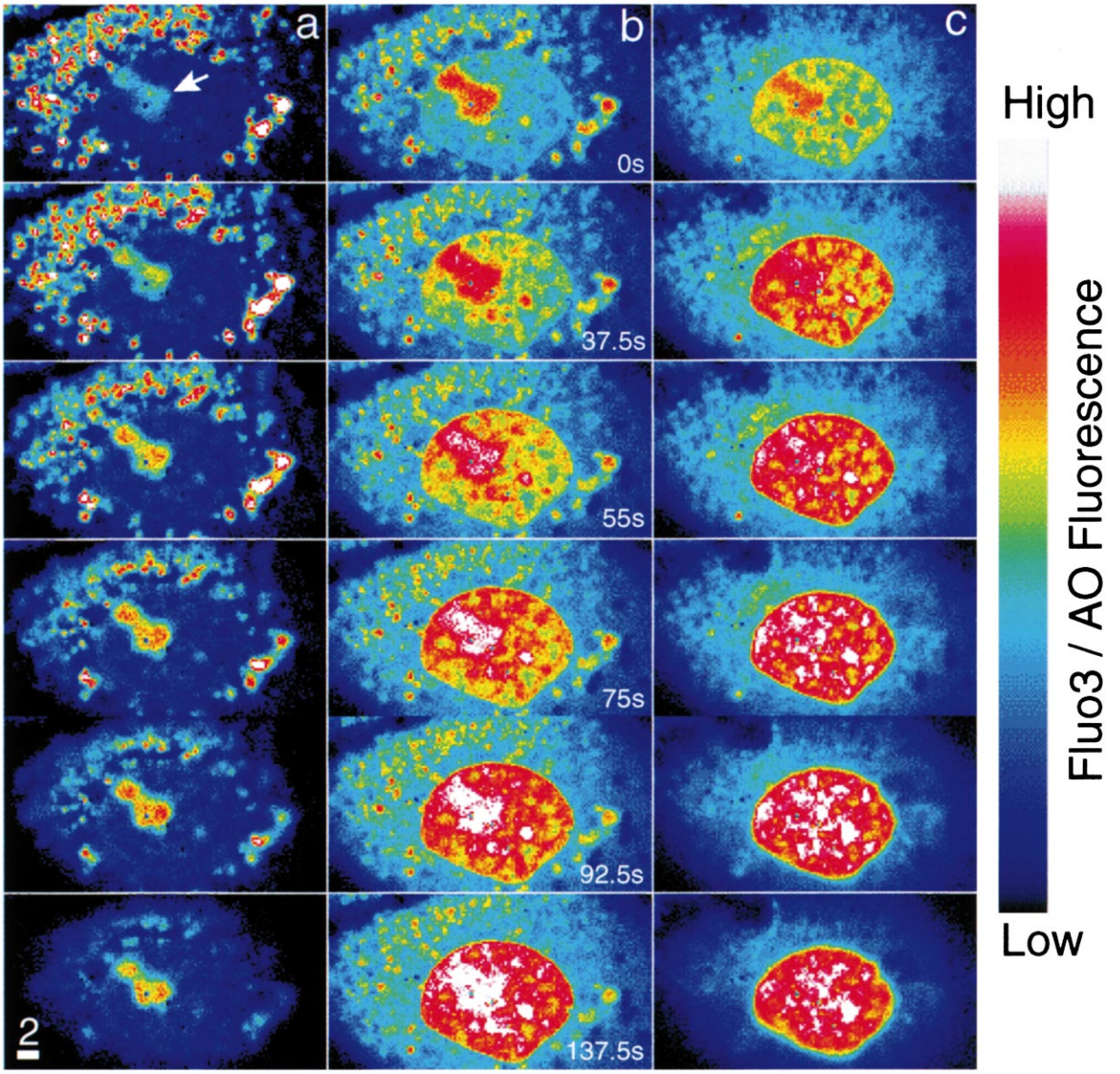

Fig. 1. Ca^{2+} released from the nuclear invagination after PDA stimulation. Panel a shows 6 optical sections collected by a confocal microscope at 0.5-μm intervals from the base (top panel) to the top of a fluo-3 loaded C6 glioma cell. Note the nuclear tubular structure inside the nucleus (arrow in white). The C6 cell was then stimulated with PDA (1 ng/ml, added at 22.5 s) at room temperature and the changes in fluo-3 fluorescence were recorded at 2.5-s intervals. Panel b shows such temporal and spatial changes at the time indicated. After the Ca^{2+} experiment, the voltage of the detector was lowered until no fluo-3 signals could be captured and AO (final concentration 0.1 μg/ml) was then added. Panel c shows a series of optical sections corresponding to the ones in panel a. Note that the chromatin formed a mesh-like matrix in the nucleus. The scale bar represents the cell dimension in μm and the color palette represents the fluorescence intensity.

amine 123 (final concentration 12.5 μg/ml) at room temperature for 10 min. After extensive washing, fluorescence was determined as described previously. At the end of the experiment, the voltage of the photomultiplier detector was lowered again and acridine orange (final concentration 0.1 μg/ml) added to study the distribution of chromatin. Images were processed by the ImageSpace software.

3. Results and discussion

Using confocal imaging, we investigated the source of nuclear Ca^{2+} in fluo-3 loaded C6 cells. Fig. 1a shows 6 optical sections of the cell at 0.5-μm intervals. In the cytosol, there were many hot spots, presumably due to the compartmentation of fluo-3 in organelles [18]. Visually most important is a tubule, seen in the sections as a highly fluorescent 'channel-like' structure in the nucleus (Fig. 1a, white arrow). Earlier work has demonstrated that phorbol ester, an activator of protein kinase C, evoked Ca^{2+} increases in C6 cells [19]. In our experiments, we therefore challenged the C6 cells with phorbol 12,13-diacetate (PDA) to study the source of nuclear Ca^{2+}. As shown in Fig. 1b, addition of PDA (1 ng/ml) at 22.5 s produced Ca^{2+} rise in both the nucleus and cytosol. In the cytosol, the fluo-3 fluorescence increased gradually with a concomitant decrease in the number of hot spots. However, no Ca^{2+} rise originated at the plasma membrane was found suggesting that internal Ca^{2+} mobilization was more important in the PDA-mediated Ca^{2+} activity. In the nucleus, PDA evoked an increase in $[Ca^{2+}]$. The rise of Ca^{2+} seemed to start from the inner part of the tubule and the NE nearby and Ca^{2+}

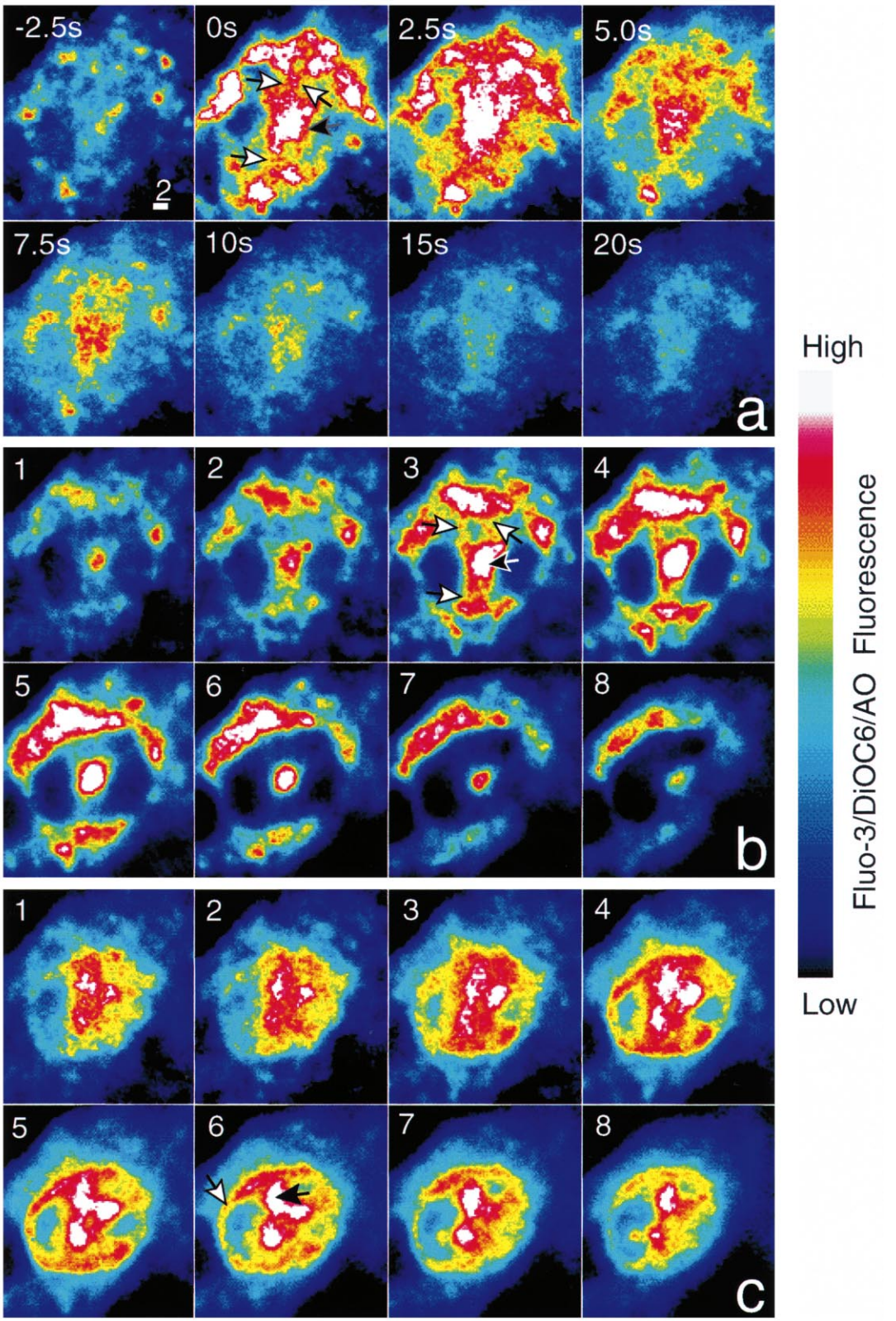

Fig. 2. Ca^{2+} released from the nuclear invagination after PMA stimulation. Panel a shows a series of images of fluo-3 loaded C6 glioma cells before and after PMA stimulation (1 ng/ml, added at −1 s). Note the Ca^{2+} rise in the specialized region (arrow in black) connected with 'filament-like' structures (arrows in white) in the nucleus. After the Ca^{2+} experiment, the voltage of the detector was lowered until no fluo-3 signals could be captured and DiOC6(3) (final concentration 0.01 μM) was then added. Panel b shows a series of optical sections of 0.5-μm intervals along the *Z*-axis (plate 1, near the base of the cell). Note the tubular structure traverses the nucleoplasm (arrow in black), contacting the envelope at plate 8. 'Filament-like' structures (arrows in white) are also visible. At the end of the experiment, the voltage of the detector was lowered again and acridine orange (AO) (final concentration 0.1 μg/ml) added. Panel c shows a series of optical sections corresponding to the ones in panel b. Note that the chromatin formed an annulus around the tubular structure. The scale bar represents the cell dimension in μm and the color palette represents the fluorescence intensity.

then spread throughout the nucleus (Fig. 1b). This result is consistent with our previous observation in C6 cells that the NE is a functional nuclear Ca^{2+} store and the enveloped Ca^{2+} is mainly released into nucleoplasm [20]. However, it has recently been indicated that fluo-3, when associated with histone/chromatin, emits stronger fluorescence than that in the cytosol even when the [Ca^{2+}] is clamped [21]. Therefore, the nuclear Ca^{2+} signals in Fig. 1a,b might be the result of the binding of fluo-3 to histone/chromatin. To test this possibility, we employed acridine orange (AO) to determine the distribution of chromatin in the nucleus of the same cell. Fig. 1c represents confocal sections corresponding to the ones in Fig. 1a but with AO staining. It is clear that chromatin formed a mesh-like matrix in the nucleus and at the same time crowded around the tubule. These images suggest that the PDA induced increase in fluo-3 signal in the nuclear tubular structure is not an artefact of non-specific binding of fluo-3 to the histone/chromatin. This conclusion is based on (i) that the morphology of the strong fluo-3 signals in Fig. 1a did not form a mesh-like matrix; (ii) that in terms of area coverage, the tubular structure labelled with fluo-3 was smaller than that with AO labelling; (iii) that stronger AO signals were always found outside the boundary of the tubular structure (Fig. 1c, areas in white around the tubular structure); (iv) that Ca^{2+} spread outwardly from the center of the tubular structure to the nucleoplasm (Fig. 1b); and (v) that no movement of chromatin was observed over a time scale of 2–3 h [22]. Our results therefore indicate that Ca^{2+} was transported from substructures in the invaginated tubules, possibly the NE into the nucleus after PDA stimulation.

To verify this conclusion, we conducted another experiment with a more potent phorbol ester, phorbo-12-myristate-13-acetate (PMA), and determined the morphology of the ER/NE subsequently with DiOC6(3), a vital fluorescent probe for intracellular membranes [23]. Fig. 2a shows the temporal and spatial changes of fluo-3 fluorescence before and after PMA stimulation. Before activation, there were hot spots with a high level of fluo-3 fluorescence in the cytosol and nucleus. After the addition of PMA (1 ng/ml), a Ca^{2+} spike was immediately observed (Fig. 2a). Similar to our previous observation (Fig. 1), Ca^{2+} was released from the hot spots (Fig. 2a: 0 s, areas in white) simultaneously in the cytosol and the nucleus but again no Ca^{2+} rise originating at the plasma membrane was observed. The rapid increase and decrease in the fluo-3 fluorescence in the nuclear tubule upon a stronger stimulation by PMA further suggest that the 'channel' plays a role in regulating nuclear Ca^{2+}. Detailed examination from Fig. 2a also reveals the existence of 'filament-like' structures (Fig. 1a: 0 s, arrow in white) which linked the nuclear hot spot with the nucleo-cytoplasmic interface. This further suggests the physical existence of the tubules in the nucleus.

Next, the morphology of the ER/NE was determined by DiOC6(3). After decreasing the sensitivity of the photon-multiplier until no fluo-3 signal was obtained, DiOC6(3) was added and the ER/NE examined. Fig. 2b depicts 8 consecutive sections of the DiOC6(3) treated cell with a vertical displacement of 0.5 μm. In these images, the nucleus (see below) appeared dark and it was surrounded by the highly-fluorescent ER/NE. Similar to our previous results, a tubular structure was seen in the nuclear region as a highly fluorescent disc (Fig. 2b, arrow in black) in each of the optical sections. Moreover, 'filament-like' connections (Fig. 1b, arrow in white) are also clearly visible (Fig. 2b: 2–5). From these images, it is conceivable that a vertical tubular structure traversed the nucleoplasm with the support from the 'filaments' and formed a connection to the top of the NE (Fig. 2b: 8). The nuclear hot spot probed by fluo-3 (Fig. 2a) was almost identical to the one labelled with DiOC6(3) (Fig. 2b) in terms of shape and location. These observations thus confirm again the existence of a membrane-bound tubular structure inside the cell nucleus.

Similar to our previous approach, AO was used to study the distribution of chromatin inside the nucleus. Sections corresponding to the ones in Fig. 2b but with AO staining are shown in Fig. 2c. In these images, both NE (arrow in white) and chromatin (arrow in black) can be visualized. Interestingly, the region of the tubular structure was devoid of nucleic acid but was surrounded by an annulus of chromatin. To have a better visual impact, we employed the DiOC6(3) and AO dataset in Fig. 2 to construct 3-D images with different rotation angles (Fig. 3). In these merged reconstructions, green represents DiOC6(3) signals while blue corresponds to AO fluorescence. It is clear in these reconstructions that the tubular structure penetrated the chromatin.

Membrane-bound tubular structures in the cell nucleus can be formed by a single- or double-membraned invagination. If the nuclear tubular structure is double-membraned, we should be able to see cytosolic organelles inside the lumen of the nuclear 'channels'. It has been demonstrated that the nucleus is surrounded by many mitochondria [24]. Using rhodamine 123 to label the mitochondria [25], we were able to locate the mitochondrial signals inside the nucleus. As shown in Fig. 4a, rhodamine signals were seen in the peri- as well as the central-nuclear region. When compared with the AO image, the rhodamine signals were definitely inside the nucleus and were surrounded by chromatin (Fig. 4b). These results thus indicate that the tubular structure is a double-membraned invagination with cytosolic organelles inside in agreement with previous studies [26,27]. Taken together, our results indicate: (i) that a tubular structure is present in the nucleus of C6 glioma cells. The orientation of the 'channel' varied from cell to cell (Figs. 1, 2 and 4); (ii) that the nuclear extension is likely a double-membraned structure largely through the invagination of the NE; (iii) that the nuclear invagination contains a core continuous with the cytoplasm since rhodamine signals indicative of mitochondria were found inside the nucleus; (iv) that the nuclear extension was usually surrounded by chromatin matrix (Figs. 2 and 4); (v) that Ca^{2+} can be released into the nucleoplasm through the nuclear structure after stimulation with phorbol ester PMA or PDA (Figs. 1 and 2).

In the past, it has been accepted that diffusion of cytosolic Ca^{2+} into the nucleus through the nuclear pore complexes is the basis for the synchronous Ca^{2+} activity in the nuclear and cytosolic compartments [14]. This hypothesis was based on the view that the nucleus is an approximate sphere surrounded by cytoplasm. Now, many lines of evidence indicate that the regulation of nuclear Ca^{2+} is much more complicated [9–11].

The nuclear invaginations have been found in many cell types of different origins [26]. With the use of transmission electron microscopy and confocal microscopy, it has been demonstrated that these invaginations are double-membraned and bear many NPC [26]. The lumen of these 'channels' contains cytosolic organelles [26]. Moreover, it was shown that the nuclear extensions are often associated with chromatin or

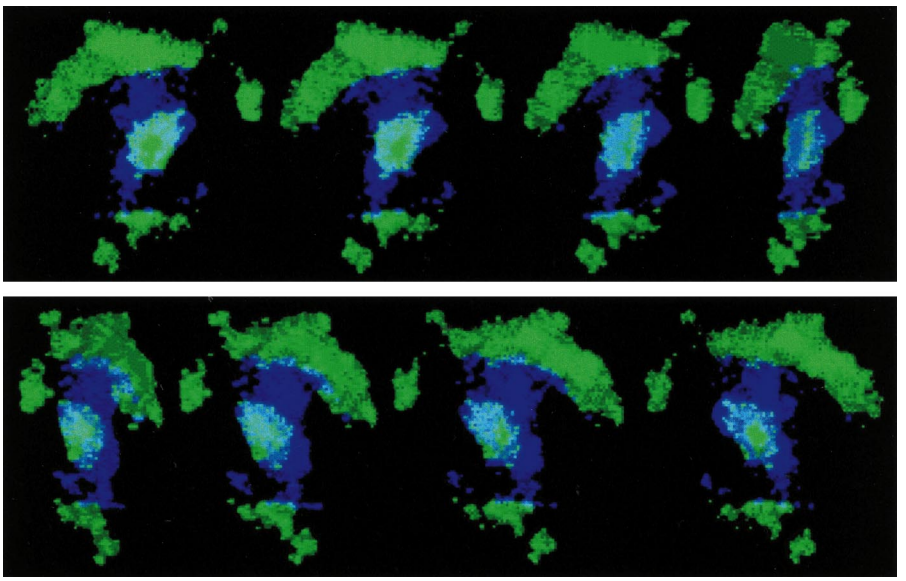

Fig. 3. The 3-D reconstruction of a nuclear tubular structure labeled with DiOC6(3) recognizing the nuclear envelope in a C6 glioma cell. The dataset in Fig. 2b,c were used to construct 3-D images. The sections nearest the base and top of the cells were discarded. Images across the nucleus with the DiOC6(3) and AO signals were reconstructed (with an increment of 20° rotation angle). In these merged reconstructions, green represents DiOC6(3) signals and blue corresponds to the AO signals. Upper panel: view from the bottom of the cell; lower panel: view from the top. Note the tubular structure ran through a hole in nucleic acid staining.

nucleoli [26,27]. Similar to these findings, our results also demonstrate the presence of nuclear invaginations in C6 cells. Our work further indicates that Ca^{2+} was released in the nucleus through these invaginations after PMA or PDA stimulation. That stimulation of cells by PMA to mimic the receptor-mediated activation of protein kinase C (PKC) is a well known phenomenon [28]. At present, we do not know the mechanism by which PMA releases Ca^{2+} inside the C6 cells nor the role of PKC in this cellular activity. However, results in NIH 3T3 cells indicate that PMA released Ca^{2+} by reducing the capacity of Ca^{2+} stores possibly through the modification of the structure of ER [29].

Accumulated evidence indicates that the ER and NE are functional Ca^{2+} stores [16,20,30] and IP3 receptors are located on the ER and the inner nuclear membrane [16,31,32]. It is therefore possible that the double-membraned invagination acts as a core in continuity with the cytosol and thereby facilitating the release of Ca^{2+} from the NE into the nucleoplasm by an unknown mechanism. The close association of the nuclear invaginations and chromatin/nucleoli could be physiologically important. Teleologically speaking, the invaginations now serve as 'tunnels' to transport many molecules, including Ca^{2+} which plays an important role in regulating gene expression [2], from the cytosol to the cell nucleus in a rapid and efficient way.

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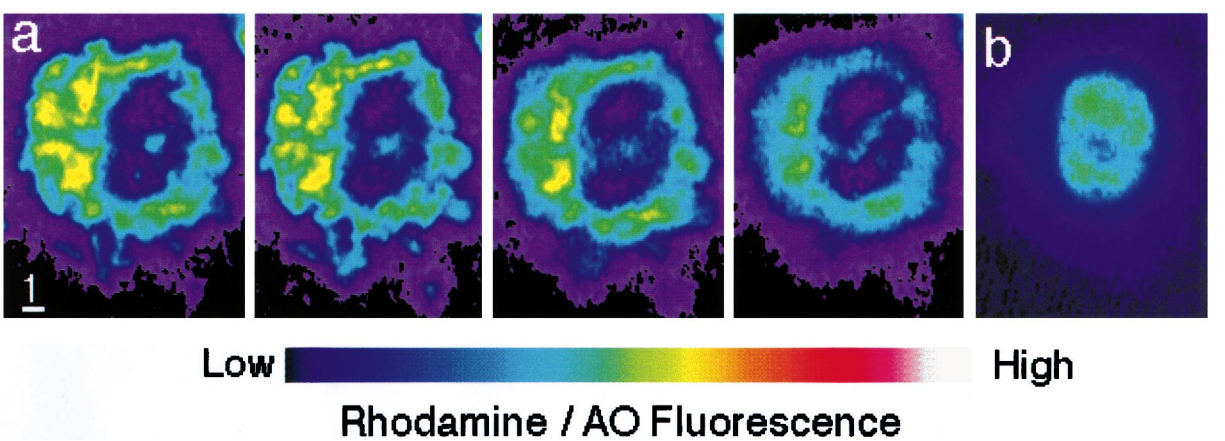

Fig. 4. Nuclear tubular structure is derived from an invagination of the double-membraned nuclear envelope. Resting C6 cells were labelled with rhodamine 123 (12.5 μg/ml) for 10 min. After extensive washing, rhodamine signals were determined by confocal microscopy. Panel a shows a series of optical sections of 0.5-μm intervals along the *Z*-axis. Subsequently, the cell received AO staining as previously described and the optical section corresponding to the third plate was recorded (panel b). Note the rhodamine 123 inside the cell nucleus and chromatin on the boundary of the nuclear tube. The presence of the rhodamine signals inside the nucleus suggest that nuclear tubular structure is derived from an invagination of the double-membraned nuclear envelope. Bar=1 μm.

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