Observations of a certain arrangement in the nucleolar chromatin after continuous ethidium bromide treatment

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Summary. This work is an ultrastructural and cytochemical study of a structure observed in the nucleolus of Allium cepa meristematic cells after nucleolar disgregation, by a continuous treatment of 12 h with ethidium bromide. The ultrastructural and cytochemical data allow us to consider this structure as the intranucleolar chromatin collapsed by the effect of the drug.

Ethidium bromide (EB) is a phenantridine which intercalates into the double helical polinucleotides, thus altering their secondary structure[2–4]. The factors implied in the interaction between EB and the double-helical DNA have been well studied in the last years. EB binds to nucleic acids or chromatin mainly in 2 ways: a) a strong binding in which the dye molecule is intercalated between the base pairs of the double-helical nucleic acids, and b) a whole electrostatic binding to the outer phosphate groups[5,6]. Recently[7] it has been demonstrated that EB intercalates mostly into the DNA in the nucleoli. In Allium cepa, the EB has an inhibitory effect on the breakdown of the nuclear envelope in prophase[8] and produces an altered evolution of mitosis in these cells[9]; it also brings about the appearance of a certain structure in the mitochondria[10], enlargement of the perinuclear space and an increase in the number of pores in the nuclear envelope[11], but the most striking effect is observed in the nucleolus. EB strongly alters the nucleolar ultrastructure[12], by producing a segregation of the nucleolar components and later on a degranulation. We have here studied the effect of a continuous EB treatment on the nucleolar chromatin in meristematic cells of Allium cepa.

Material and methods. Meristem roots of Allium cepa bulbs were grown in the dark at a constant temperature of 15 °C ± 0.5 in tap water which was renewed every 24 h and aerated continuously by bubbling at 10–20 ml min^{-1}. The roots, still attached to the bulbs, were transferred to the EB (100 µg ml^{-1}) under the same conditions used for growing for 12 h after beginning the treatment. The roots were fixed as follows. Conventional fixing: Fixation in 3% glutaraldehyde in a 0.025 M cacodylate buffer, pH 7.0, for 1 h at room temperature, and postfixation in 1% osmic acid for 1 h in the same buffer. For preferential stains roots were fixed in 2.% glutaraldehyde in Sorensen's phosphate buffer, pH 7.3, for 1 h at room temperature. The roots were dehydrated with graded concentrations of ethanol, passed through propylene oxide and embedded in Epon 812. Staining: Sections were stained with 1% uranyl acetate in absolute ethanol for 30 min at room temperature and poststained with lead citrate according to Venable and Gogeshall[13]. Preferential stains. 1. Deoxyribonucleoproteins: Ultrathin sections were hydrolyzed in 5 N ClH for 25 min and floated on a solution of osmium ammine prepared according to Cogliatti and Gautier[14]. 2. Ribonucleoproteins: Ultrathin sections were stained by the uranyl acetate-EDTA-lead citrate sequence according to Bernhard[15]. Enzymatic digestions: Roots were fixed in 1.6% glutaraldehyde in Sorensen's buffer, pH 7.0, for 15 min to 1 h at 4 °C and embedded in GMA, according to Leduc and Bernhard[16]. Sections were taken on Marinozzi's plastics rings, and then floated on the enzyme solutions at 37 °C for 2 h. (Parallel sections of control roots were floated on similar solutions but without the enzyme.) Then the sections were floated on bidestilled water several times at 37 °C for 30 min, placed on formvard-coated grids and there stained with uranyl acetate and lead citrate. The enzyme solutions used were: Deoxyribonuclease (DNase I Sigma) 0.1% DNase in $MgCl_2$, 0.001 M, pH 6.8. Rinonuclease (Koch Light): 0.1% RNase in this buffer 0.2, pH 7.2 + 0.01 M $MgCl_2$. Trypsin (type III Sigma): 0.03% Trypsin in distilled water, pH 8.0, adjusted with 0.01 M NaOH. Observations were carried out in a Philips EM 300.

Results and discussion. As was previously described[12], EB produces a strong alteration of the nucleolar ultrastructure in Allium cepa. After 12 h of treatment, the nucleolus has disappeared almost completely in most of the cells, but the space previously occupied by this organelle remains free surrounded by the perinucleolar chromatin (figure 1). Within this free space, appears a structure with the same contrast as chromatin, showing continuity with the perinucleolar chromatin. In favorable sections one can observe 2 of these structures in each nucleus (figure 2), that is, as many structures as nucleolar organizing regions (NOR) in these cells. It shows a similar aspect to the NOR previously described in Allium cepa[17,18]. It is composed of groups of fibrils with a high contrast, which are stained by osmium ammine (figure 3) but not by EDTA, and which resist the action of trypsine (figure 4) and RNase (figure 2) the results obtained from DNase digestions are not clear; all of which strongly suggests that they correspond to chromatin. These groups of fibrils are very similar in structure to the condensed chromatin zones described in the NOR in Allium cepa an-

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thers[18] and meristematic cells[17], but they have a more irregular distribution than in controls and seem to be clustered at some points (arrows) (figure 4). In among these fibres, one can observe another type of fibrils (80 Å) with a lower contrast than the former (arrows) (figure 1) which are not contrasted by osmium ammine (figure 3) but contrast with EDTA (figure 5) and which partially resist the digestion with trypsin (figure 4) but not with RNase (figure 2) and which would therefore have a ribonucleoproteic nature.

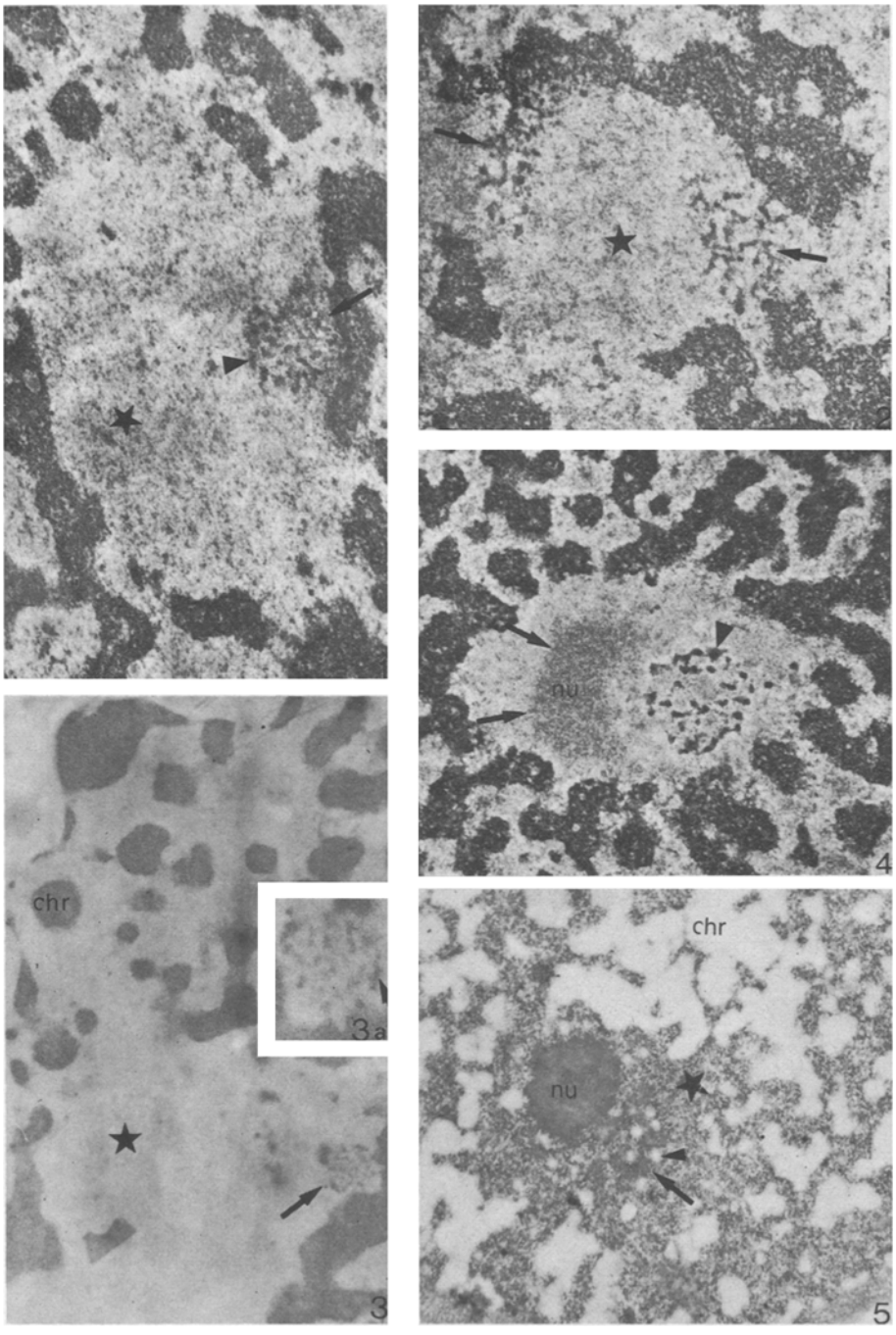

Fig. 1–5. Nucleus of meristematic cells of A. cepa treated for 12 h with Ethidium bromide. – Fig. 1. Glutaraldehyde fixation. Aspect of the nucleolus after a continuous 12-h-treatment of EB. The nucleolar mass has disappeared, but the perinucleolar chromatin does not occupy the nucleolar space (*). The NOR remains bound to the perinucleolar chromatin. In this region, the fibres identified as intranucleolar chromatin (arrowshead) and the nucleolar material filling the NOR (arrows) are clearly observable. × 25,650. – Fig. 2. After Rnase digestion, the nucleolar material filling the NOR mesh disappears, but the NOR remains. In this case the 2 NORs bound to perinucleolar chromatin are observable (arrows). × 24,300. – Fig. 3. With osmium ammine the fibrils corresponding to the intranucleolar chromatin (arrows) are stained in the same way as the nuclear chromatin, but the nucleolar material associated to them is not. × 23,600. 3a, × 47,900. – Fig. 4. After trypsine digestion, the fibrils of the intranucleolar chromatin remain and show the typical aspect with zones where the chromatin fibres are collapsed (arrowhead). A remnant of the nucleolus which resists trypsine digestion is also observed (arrow). × 15,960. – Fig. 5. The EDTA technique preferentially reveals RNP carrying nucleolar material (arrows) filling the NOR mesh. The intranucleolar chromatin (arrowshead) corresponding to the NOR mesh is bleached in the same way as the nuclear chromatin. A remnant of the nucleolus is stained (Nu). × 15,000.

The ultrastructural and cytochemical analyses lead us to suppose that this structure would correspond to the NOR undergoing a collapse within the lacunar areas of the nucleolus by the action of the drug, as has been reported with other drugs which interact with nucleolar activity[19,20]. After the nucleolar degranulation and later disappearance of the nucleolus as an organized structure, the NOR would preserve its conformation, in the same way as the perinucleolar masses of condensed chromatin do, not occupying the space previously filled by the nucleolus. This would indicate that continuous EB treatment may produce a rigidity in the nucleolar as well as in the extranucleolar chromatin, as other authors[21-23] and we[9] have previously reported. The ribonucleoproteic structures with a lower contrast, which appear filling the spaces in among the groups of nucleolar chromatin fibres, would correspond to remnants of altered nucleolar material related to the fibrillar zone[12] which would be trapped by the NOR's mesh. The collapse of the intranucleolar chromatin areas is a morphological effect of the inhibition of DNA-directed RNA synthesis different to the appearance of fibrillar bodies.

These bodies, which appear in Allium cepa[24,25] and also in animal cells[26] after RNA synthesis inhibitor treatments, have also been described as a constant component of the nucleolus[27], and seem to correspond to dispersed chromatin[27,28]. Under EB these bodies do not show a collapsed aspect, as does the intranucleolar chromatin, perhaps because of their high protein content[24,25,27] which would make them in accessible to the EB, or perhaps because, as we postulated previously[24,25], they are inactive organelles not involves in active cellular metabolism.

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Inhibition by verapamil of ionophore-mediated calcium translocation[1]

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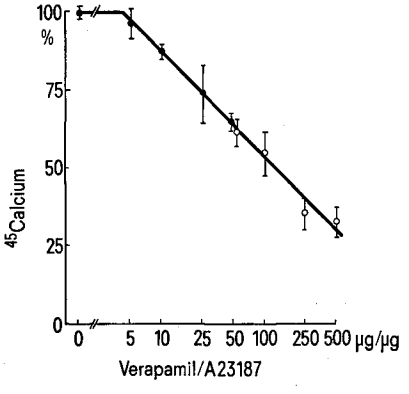
Summary. Verapamil and other organic calcium-antagonists inhibit the A23187-mediated translocation of calcium from an aqueous into an organic phase.

Organic calcium-antagonists such as verapamil and D600 are thought to inhibit the entry of calcium into cells across the plasma membrane, and are currently used to investigate the participation of such a calcium influx in calcium-dependent functional processes, e.g. contraction and secretion[2,3]. However, the precise mode of action of these drugs, at the physicochemical level, remained to be elucidated. It is here proposed that organic calcium-antagonists directly interfere with the calcium-binding sites of ionophores.



Effect of verapamil upon A23187-mediated calcium translocation. The amount of ^{45}calcium recovered in the immiscible phase is expressed in percent of the appropriate mean control value found in the absence of verapamil, and is shown as function of the ratio between verapamil and ionophore concentrations (µg/µg) in the initial organic phase. The experiments were performed in the presence of A23187 at concentrations of 20 (closed circles) and 2 (open circles) µM in the initial organic phase. Each point (± SEM) refers to 3 individual measurements. The control values for the concentration of calcium in the immiscible phase averaged 140.4 ± 2.0 and 2.2 ± 0.1 nM at ionophore 20 and 2 µM respectively, the experiments being carried out at a 11 µM concentration of calcium in the initial aqueous phase.

A small volume (0.2 ml) of Hepes buffer (25 mM; pH 7.0) containing Na^+ 123, K^+ 5 and Cl^- 115 mEq/l and ^{45}calcium (10 µCi/ml) was vigorously mixed for 1 min at room temperature with an equal volume of a mixture of toluene-butanol (7/3, v/v) containing, as required, the ionophore A23187 (Eli Lilly, Indianapolis) and various organic calcium-antagonists. The supernatant immiscible phase was then examined for its radioactive content.

The ionophore A23187 provokes a dose-related and reversible translocation of ^{45}calcium from the Hepes buffer into the immiscible phase. In addition to previous studies[4], extensive investigations on the modulation of ^{45}calcium translocation by temperature, mixing time, volume of reagents, pH, and concentration of ionophore, monovalent cations, calcium and other divalent cations established the validity of the present system as a model for the study of the ionophoretic properties of A23187 (unpublished observations).

As shown in the figure, verapamil inhibited the A23187-mediated translocation of calcium. The degree of inhibi-

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