Chloral hydrate disrupts mitosis by increasing intracellular free calcium

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Summary

In examining how chloral hydrate affects mitosis, we found that extracellular application of 0·1 % chloral hydrate produced an abrupt rise in cytosolic free Ca^{2+}. Digitized fluorescence microscopy of Fura-2-loaded, mitotic and interphase PtK cells revealed that Ca^{2+} rose 15 s after chloral hydrate application, peaked within 1 min at a concentration two- to sevenfold above the basal level and then slowly dropped. Bathing cells in 0·1 % chloral hydrate caused metaphase spindles to shorten, starting in 1–2 min, and inhibited spindle elongation without affecting chromosome-to-pole movement during anaphase, as determined by phase-contrast observation of living cells. Spindle elongation and chromosome movement were unaffected by intracellular injection of 7·5 % chloral hydrate. Extensive mitotic microtubule breakdown occurred after cells were bathed for 7 min in 0·1 % chloral hydrate, while interphase microtubules were unaffected as determined by immunofluorescence. The chloral hydrate-induced microtubule breakdown and metaphase spindle shortening were prevented by 10 mM-$CoCl_2$, which has previously been shown to block Ca^{2+} influx and to stabilize microtubules *in vitro*. These results imply that disruption of mitotic spindle function and structure by chloral hydrate is due to a rise in cytosolic free Ca^{2+}, and also indicate that mitotic microtubules are more Ca^{2+}-labile than interphase microtubules.

Key words: anaesthetic, spindle structure, cobalt chloride.

Introduction

Chloral hydrate is a sedative–hypnotic drug and a metabolite of the widely used industrial chemical, trichloroethylene. It is also a known mutagen (Russo *et al.* 1984; Kafer, 1986), which acts by disrupting mitosis (Mercer & Morris, 1975; Ates & Sentein, 1978; Mole-Bajer, 1967). The disruption is due to mitotic spindle collapse as determined by electron microscopy of cells bathed in dilute solutions of chloral hydrate (Mole-Bajer, 1967). When applied in a very narrow concentration range, chloral hydrate inhibits spindle elongation without affecting chromosome-to-pole movement during anaphase in grasshopper spermatocytes (Ris, 1949).

The effects of chloral hydrate on mitosis may involve Ca^{2+} regulation. Other general anaesthetics, such as C_5 to C_{10} alcohols and chloroform, cause an increase in intracellular free Ca^{2+} (Vassort *et al.* 1986). The possibility that chloral hydrate raises intracellular Ca^{2+} was first proposed by Dunlap (1977) to explain how chloral hydrate produces deciliation of *Paramecium caudatum*. The rise in intracellular Ca^{2+} might occur *via* inhibition of the plasma membrane Ca^{2+}/Mg^{2+}-ATPase, since chloral hydrate reversibly inhibits this enzyme *in vitro* (Bergesse *et al.* 1983). This ATPase functions to pump Ca^{2+} out of the cell, thus fine-tuning the intracellular free calcium concentration (Carafoli & Penniston, 1985; Schatzmann, 1982). Vasopressin and insulin, agents that are known to cause an elevation in intracellular calcium (Berridge &

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Irvine, 1984; Lemasters *et al.* 1987), inhibit this plasma membrane calcium pump (Lin *et al.* 1983; Pershadsingh & McDonald, 1981).

Alterations in levels of cytosolic free calcium may be involved both in microtubule lability and in the regulation of mitosis. Microtubules in intact cells (Kiehart, 1981; Izant, 1983), in isolated spindles (Salmon & Segall, 1980; Salmon, 1982) and assembled *in vitro* (Marcum *et al.* 1978; Rebhun *et al.* 1980) are labile to micromolar concentrations of Ca^{2+}. The rate of microtubule breakdown is directly related to Ca^{2+} concentration. Physiologically elevated Ca^{2+} levels have been suggested as being involved in microtubule turnover (Fuller & Brinkley, 1976; Marcum *et al.* 1978; Salmon & Segall, 1980). This suggestion is given credence by the recent finding of a sudden, transient rise in calcium to 500–800 nM-Ca^{2+} at the onset of anaphase (Poenie *et al.* 1986). This finding also emphasizes the role of calcium in the regulation of mitosis. Further evidence for the importance of Ca^{2+} in the metaphase–anaphase transition is that metaphase PtK cells enter anaphase more rapidly after injection of 1–10 μM-$CaCl_2$ (Izant, 1983) and that the metaphase–anaphase transition is prevented by blocking Ca^{2+} influx (Hepler, 1985). Calcium may also be involved in local regulation within the spindle, since the spindle contains many vesicles that sequester calcium (Hepler & Wolniak, 1984). In addition, the distribution of calcium (Ratan *et al.* 1986) and of calmodulin (De Mey *et al.* 1980; Vantard *et al.* 1985) within the spindle changes as the cell progresses through mitosis.

In the present study, we found a rapid rise in intracellular free calcium in PtK cells following exposure to chloral hydrate, by using the fluorescent Ca^{2+} indicator, Fura-2, and digitized fluorescence microscopy. Fura-2 was particularly useful for our purpose of studying a drug with unknown, possibly multiple, effects on the cell since it is well-established as a Ca^{2+} indicator (Grynkiewicz *et al.* 1985; Lemasters *et al.* 1987; Poenie *et al.* 1985, 1986; Ratan *et al.* 1986; Roe *et al.* 1987; Wier *et al.* 1987) and is fairly insensitive to changes in pH and Mg^{2+} within the physiological range (Grynkiewicz *et al.* 1985; Poenie *et al.* 1985). Demonstrating the role of this rise in calcium in producing the observed effects of chloral hydrate on mitosis is less straightforward. Several approaches were used. (1) Extracellular, but not intracellular, application of chloral hydrate inhibited spindle elongation, which implies that chloral hydrate was not acting directly on the spindle but *via* an intermediate such as Ca^{2+}. (2) The pattern of chloral hydrate-induced microtubule breakdown, revealed by tubulin immunofluorescence, compared favourably with Ca^{2+}-induced microtubule breakdown observed in previous studies. (3) Treatment with $CoCl_2$, which has been shown to inhibit Ca^{2+} influx (Kohlhardt *et al.* 1973) and to stabilize microtubules against Ca^{2+} (Wallin *et al.* 1977), maintained spindle structure in the presence of chloral hydrate.

Materials and methods

PtK cells, cell lines derived from rat kangaroo kidney epithelium (PtK1 from American Type Culture Collection, Rockville, MD; and PtK2, a gift from M. Berns, University of California, Irvine) were maintained in tissue culture medium: Ham's F12 nutrient mixture supplemented with 10% foetal calf serum (GIBCO, Grand Island, NY) and 0·05 mg ml^{-1} neomycin sulphate or 0·1 mg ml^{-1} kanamycin (Sigma, St Louis, MO). For anaphase rate measurements and Ca^{2+} determinations, cells were plated 2–6 days prior to use in chambers assembled from 25 mm round coverslips attached with silicone vacuum grease to 5 mm high glass cylinders (Rappaport, 1986). Anaphase rate measurements were done with cells in tissue culture medium plus 50 mM-Hepes (Sigma) covered with a thin layer of mineral oil (Squibb). Spindle structure experiments and Ca^{2+} measurements were done with cells in saline G (140 mM-NaCl, 5 mM-KCl, 6 mM-glucose, 0·6 mM-$MgSO_4$, 1·1 mM-$CaCl_2$, 1 mM-Na_2HPO_4, 1 mM-KH_2PO_4) at 34–37°C. The phosphates in saline G were replaced with 25 mM-Hepes for the $CoCl_2$ treatments.

An inverted Zeiss microscope with 40× phase-contrast optics (numerical aperture: of objective, 0·75; of condenser, 0·9) was used for the anaphase rate measurements. Temperature was maintained at 34–35°C with a Sage air-curtain incubator and monitored with a Tele-Thermometer (Yellow Springs Instrument Co., Inc.). Kinetochore separation and spindle elongation were measured directly using an ocular micrometer. At the onset of anaphase, chloral hydrate (Fisher Scientific) was applied either extracellularly in tissue culture medium or by pressure microinjection in 100 mM-Pipes, 0·1 mM-$MgCl_2$ (MI buffer).

The equipment and procedure for the Ca^{2+} measurements have been described (DiGuiseppi *et al.* 1985; Lemasters *et al.* 1987). Briefly, a video digitizing system was used to acquire pixel-by-pixel averaged fluorescence intensities of Fura-2-loaded cells observed at >450 nm when excited at 340 and 380 nm. After correction for background and autofluorescence, the ratio of fluorescence emission intensity obtained at the two excitation wavelengths (340 nm divided by 380 nm) was computed for each pixel. The Ca^{2+} concentration was computed from the mean pixel ratio for the cell using a standard curve generated by measurement of Fura-2 pentapotassium salt, Ca–EGTA buffers of known Ca^{2+} concentration. It is clear that the intracellular environment is different from that of our standard Fura-2 solutions, and calculations by others (Poenie *et al.* 1986) show that differences between the two environments can lead to a 15% error in the estimation of Ca^{2+} concentrations in cells. However, we are concerned with relative and not absolute changes, and recognize that our values may be in error by 15%. Fura-2 pentapotassium salt (Molecular Probes, Junction City, OR) (1 mM in MI buffer) was microinjected into the PtK cells for measurement of intracellular free Ca^{2+}. The microinjected interphase and mitotic cells were held at room temperature

and 4°C, respectively, for approximately 2–4 h and then warmed to 34°C for 2–10 min prior to measurement. The mitotic cells were kept cold to stop their progress through mitosis while they were being transported from the laboratory where the microinjections were done to the laboratory where the Ca^{2+} measurements were made. Measurements were done only on cells with uniformly fluorescent cytoplasm that were typical of the majority in size and had either a single, round nucleus or a bipolar spindle.

Spindle structure was examined by immunocytochemistry after several treatments: chloral hydrate, $CoCl_2$, $CoCl_2$ plus chloral hydrate, and nocodazole after $CoCl_2$ or $CoCl_2$ plus chloral hydrate. For the chloral hydrate treatment, 0·1 % chloral hydrate in Hepes-buffered tissue-culture medium or in saline G was applied to the PtK cells on coverslips for 10 min at 37°C. For the $CoCl_2$ treatment, cells were incubated 11 min at 37°C in 10 mM-$CoCl_2$. For $CoCl_2$ followed by chloral hydrate, cells were incubated for 1 min in $CoCl_2$ at 37°C and then 10 % chloral hydrate in dH_2O was added to the $CoCl_2$-containing buffer to a final concentration of 0·1 % and incubation was continued for an additional 10 min at 37°C. For the nocodazole treatment, the cells were rinsed briefly then transferred to 2·5 μM-nocodazole (Sigma) for 10 min at 37°C. The coverslips were transferred to 1 % glutaraldehyde in phosphate-buffered saline (PBS) (Osborn & Weber, 1982) at room temperature for 10 min. All of the following steps were done with PBS at room temperature. After four rinses for 5 min each, the coverslips were treated for 30 min in 0·5 % Nonidet P-40 (Sigma), rinsed briefly, treated for 15 min in 0·5 mg ml^{-1} $NaBH_4$, rinsed twice for 10 min, and once in 1 % bovine serum albumin (BSA/PBS) for 20 min. Then 25–30 μl of 1:500 (v/v) mouse anti-α-tubulin and anti-β-tubulin (Amersham Corp., Arlington, IL) was applied. After a 45-min incubation at 37°C, the coverslips were rinsed three times for 10 min in BSA/PBS. Then 25–30 μl of 1:15 (v/v) TRITC-labelled goat anti-mouse affinity-purified IgG (Jackson Immuno Research Lab., Avondale, PA) was applied followed by a 45-min incubation at 37°C. After three 10-min rinses in BSA/PBS, the coverslips were mounted on slides with a polyvinyl alcohol mounting medium (Osborn & Weber, 1982) containing 1 % propyl gallate.

Cells were selected for photography using the following criteria: (1) in the desired stage of mitosis (late anaphase or mid to late metaphase), (2) intensely stained, and (3) very flat with both poles in focus. Micrographs were taken using Kodak Technical Pan 2415 film at ASA 80 and processed with Kodak HC110 developer, dilution B for 6 min.

Results

Effect of chloral hydrate on calcium concentration

As determined by digitized fluorescence microscopy of Fura-2-loaded cells, intracellular free Ca^{2+} started to rise within 15 s and went up 2·3- to 7-fold within 1 min after extracellular application of 0·1 % chloral hydrate. Two mitotic cells had a greater than 20-fold increase in Ca^{2+}. For both mitotic and interphase cells, the Ca^{2+} then returned to a lower level but had not returned to the baseline level after 12 min. Average values for both mitotic and interphase cells are given in Table 1. The rise in Ca^{2+} concentration in response to chloral hydrate is plotted in Fig. 1 for an interphase and a mitotic cell. The rapid rise in Ca^{2+} in response to chloral hydrate is typical of all cells measured, but the peak concentration and the rate and extent of decrease after reaching the peak concentration were highly variable (Table 1). The ratioed image of the chloral hydrate-treated cells revealed an even increase in Ca^{2+} over the whole cell (not shown). Interphase cells had a higher average basal level of Ca^{2+} than did mitotic cells (Table 1).

Effect of chloral hydrate on anaphase chromosome movement

The extracellular application of 0·1 % chloral hydrate at the onset of anaphase greatly inhibited spindle elongation (Fig. 2). The spindles in buffer-injected cells increased in length by an average of 71 %, whereas chloral hydrate-bathed cells increased in spindle length by 32 %. When chloral hydrate-bathed cells were compared with cells injected with MI buffer or chloral

Table 1. *The effect of chloral hydrate on intracellular calcium*

	Interphase	Mitotic
Number of cells	6	7
Baseline concn (nM)*	164 ± 17	79 ± 14
Peak concn (nM)	567 ± 46	724 ± 132
Time to peak (s)	49 ± 20	49 ± 5
Concn at 4 min (nM)	381 ± 50	489 ± 80

Values are given as mean ± standard error.
*Concn, Ca^{2+} concentration as determined by digitized fluorescence microscopy of Fura-2-loaded PtK1 cells.

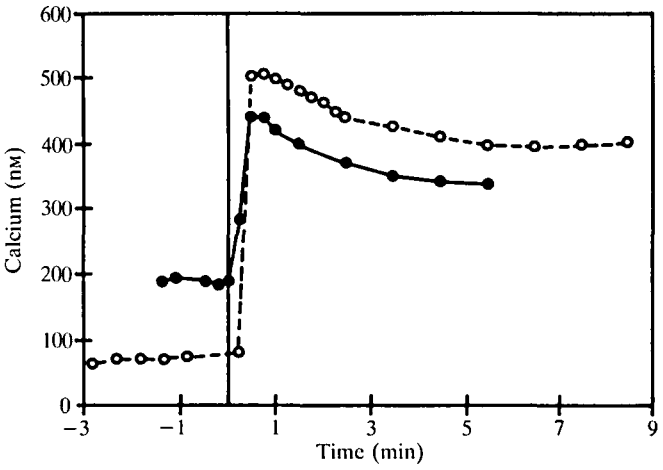

Fig. 1. Time course of Ca^{2+} concentration in response to 0·1 % chloral hydrate added at 0 time. The Ca^{2+} concentration was determined by digitized fluorescence microscopy of Fura-2-stained PtK1 cells. (O---O) A metaphase cell; (●——●) an interphase cell.

hydrate, the difference was statistically significant at the $P < 0{\cdot}01$ level as determined by two-way analysis of variance with repeated measures. However, the injection of 7·5 % chloral hydrate had little effect ($P > 0{\cdot}94$ when compared with the buffer-injected cells). Cytokinesis was unaffected by chloral hydrate.

Chromosome-to-pole movement is plotted in Fig. 3. Since kinetochore separation involves both chromosome-to-pole movement and spindle elongation, any retardation of spindle elongation will also affect kinetochore separation. Therefore, the separation due to spindle elongation has been subtracted from each of the respective curves. The rate of chromosome-to-pole movement after exposure to chloral hydrate, either applied extracellularly or injected, was not significantly different from that in the MI buffer-injected cells ($P > 0{\cdot}1$, two-way analysis of variance with repeated measures).

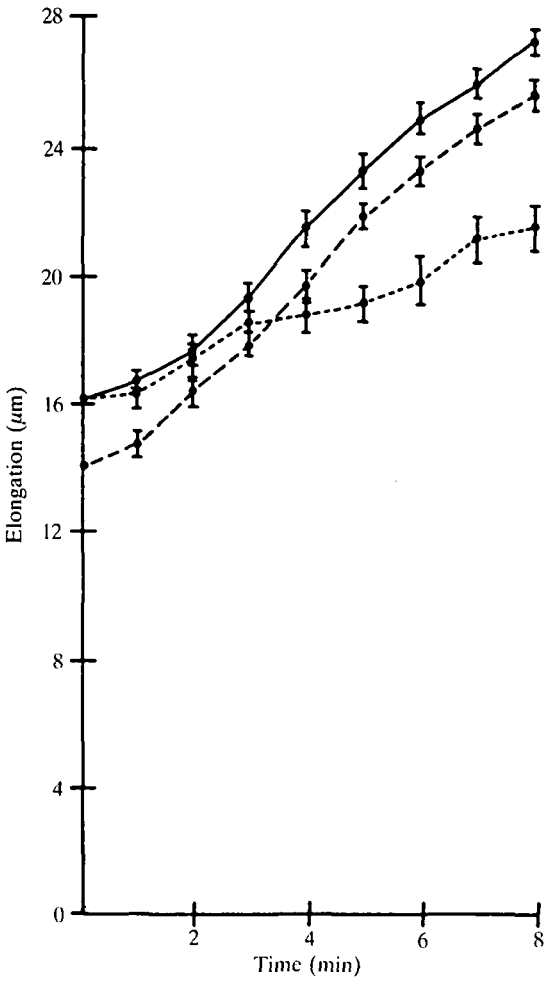

Fig. 2. Anaphase spindle elongation in PtK2 cells after treatment: (——) MI buffer microinjected (10 cells); (– – –) 7·5 % chloral hydrate microinjected (12 cells); (·····) extracellular 0·1 % chloral hydrate (7 cells). All treatments were given at the onset of anaphase. Bars represent standard error.

Effect of chloral hydrate on spindle structure

As can be seen in Fig. 4, the effect of 0·1 % chloral hydrate applied extracellularly on anaphase spindle structure after 5 min involved no apparent decrease in microtubules but interzone and astral microtubules became wavy. The comparatively long astral microtubules seen in Fig. 4B may or may not be due to exposure to chloral hydrate, since astral microtubule length is quite variable in different anaphase cells. By 10 min, astral microtubules had disappeared and there appeared to be some reduction in the number of interzone microtubules.

In living metaphase cells, the spindles started to shorten within 1–2 min after application of 0·1 % chloral hydrate and continued to shorten for approximately 3 min. As shown by tubulin immunofluorescence (Fig. 5), the astral microtubules became longer during this time period as compared to untreated late metaphase cells, which have uniformly short astral microtubules. By 10 min, astral and interpolar microtubules have mostly disappeared (Fig. 5D). When cells were placed in fresh, chloral hydrate-free tissue-culture medium at 37 °C, spindle structure returned to normal in less than 10 min (not shown).

Effect of cobalt ions and chloral hydrate on spindle structure

Pretreatment and the continued presence of 10 mM-$CoCl_2$ prevented the chloral hydrate-induced spindle collapse of metaphase cells (Fig. 6). To determine whether Co^{2+} was entering the cell and stabilizing

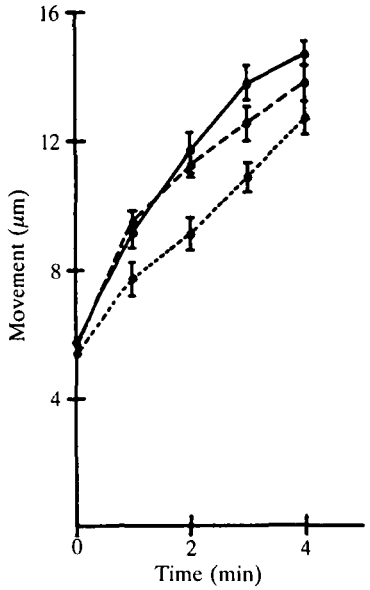

Fig. 3. Anaphase chromosome-to-pole movement in PtK2 cells after treatment: (——) MI buffer microinjected (10 cells); (– – –) 7·5 % chloral hydrate-microinjected (12 cells); (·····) extracellular 0·1 % chloral hydrate (7 cells). All treatments were given at the onset of anaphase. These are the same cells as in Fig. 2. Bars represent standard error.

microtubules before the addition of chloral hydrate, cells were rinsed prior to the addition of chloral hydrate. In this case, spindle collapse was not prevented (Fig. 6D). In addition, when cells in 10 mM-$CoCl_2$ solution were exposed to 2·5 μM-nocodazole for 10 min, microtubule breakdown was the same as in cells exposed to nocodazole without $CoCl_2$ (Fig. 7). (Note: cobalt ions stabilize microtubules *in vitro* against calcium and colchicine (Wallin *et al.* 1977). Like colchicine, nocodazole depolymerizes microtubules *in vivo* (DeBrabander *et al.* 1986)). To determine whether Co^{2+} entered the cell when chloral hydrate was added, cells were treated with $CoCl_2$ plus chloral hydrate and then exposed to nocodazole. In

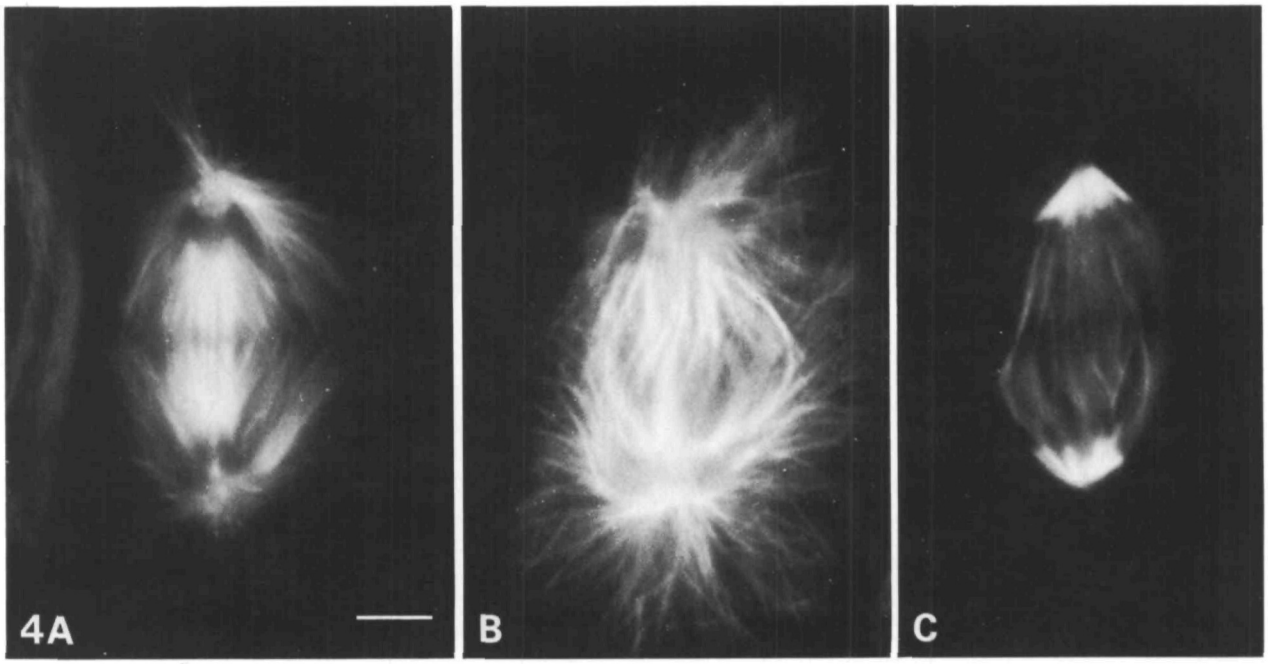

Fig. 4. Effect of 0·1 % chloral hydrate on anaphase spindle structure in three different PtK1 cells. A. Buffer only; B, after a 5 min exposure to chloral hydrate; C, after a 10 min exposure to chloral hydrate. Bar, 5 μm.

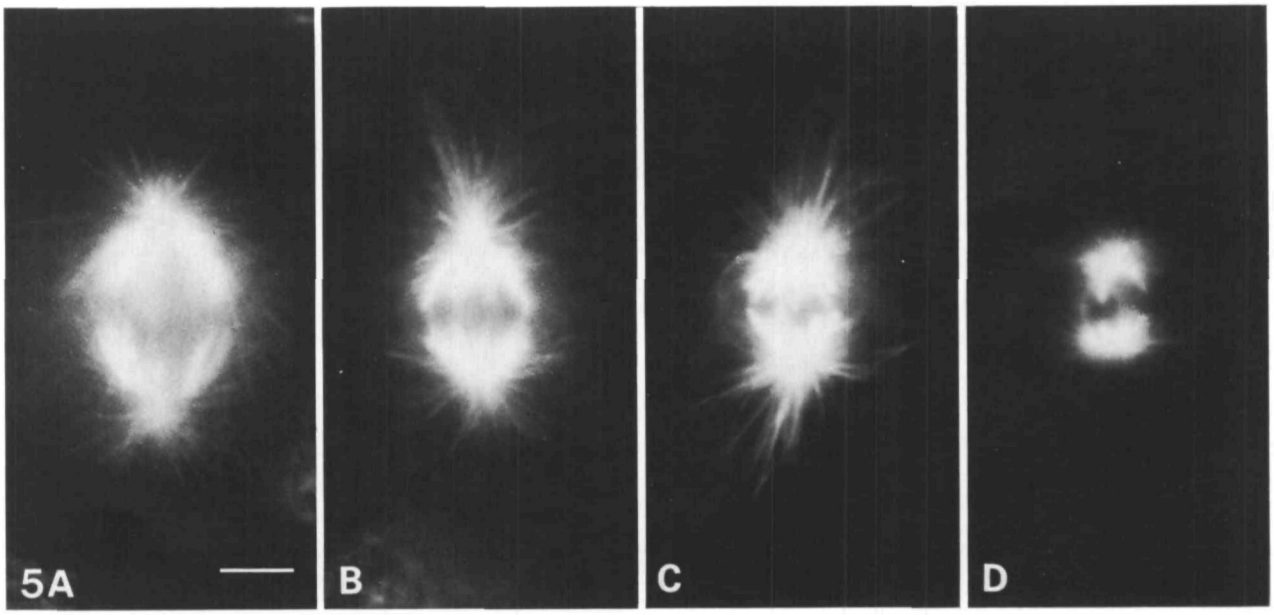

Fig. 5. Effect of 0·1 % chloral hydrate on metaphase spindle structure in four different PtK1 cells. A. Buffer only; B, after a 2 min exposure to chloral hydrate; C, after a 5 min exposure to chloral hydrate; D, after a 10 min exposure to chloral hydrate. Bar, 5 μm.

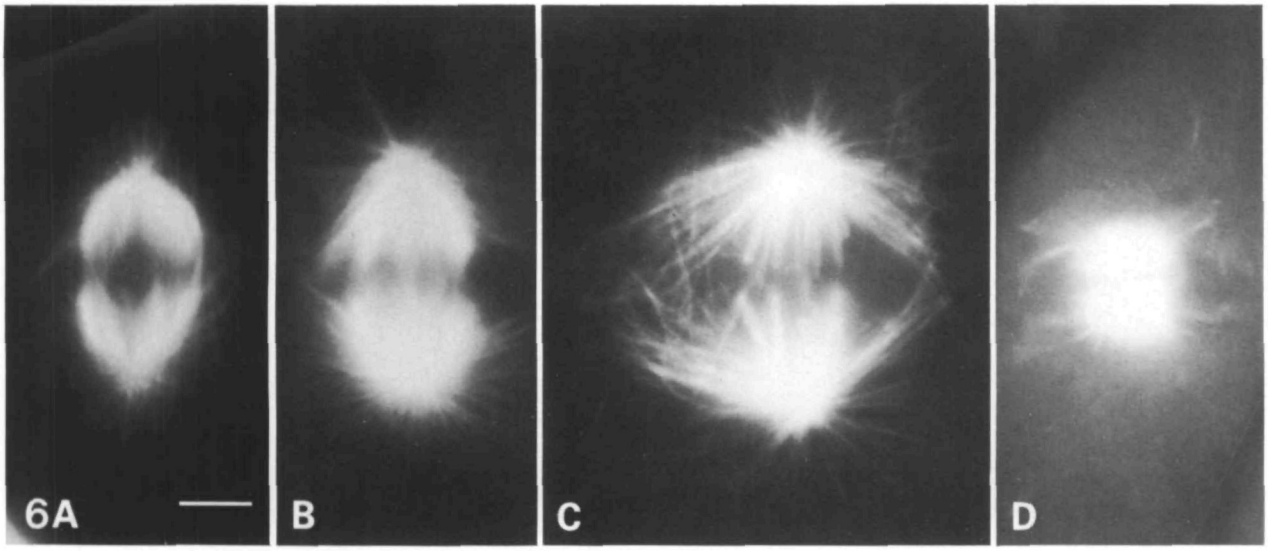

Fig. 6. Effect of 0·1 % chloral hydrate counteracted by 10 mM-$CoCl_2$ on metaphase spindle structure in PtK1 cells. A. Buffer only; B, 11 min exposure to $CoCl_2$; C, 1 min exposure to $CoCl_2$ followed by 10 min exposure to chloral hydrate plus $CoCl_2$; D, 1 min exposure to $CoCl_2$, rinsed and then chloral hydrate for 10 min. Bar, 5 μm.

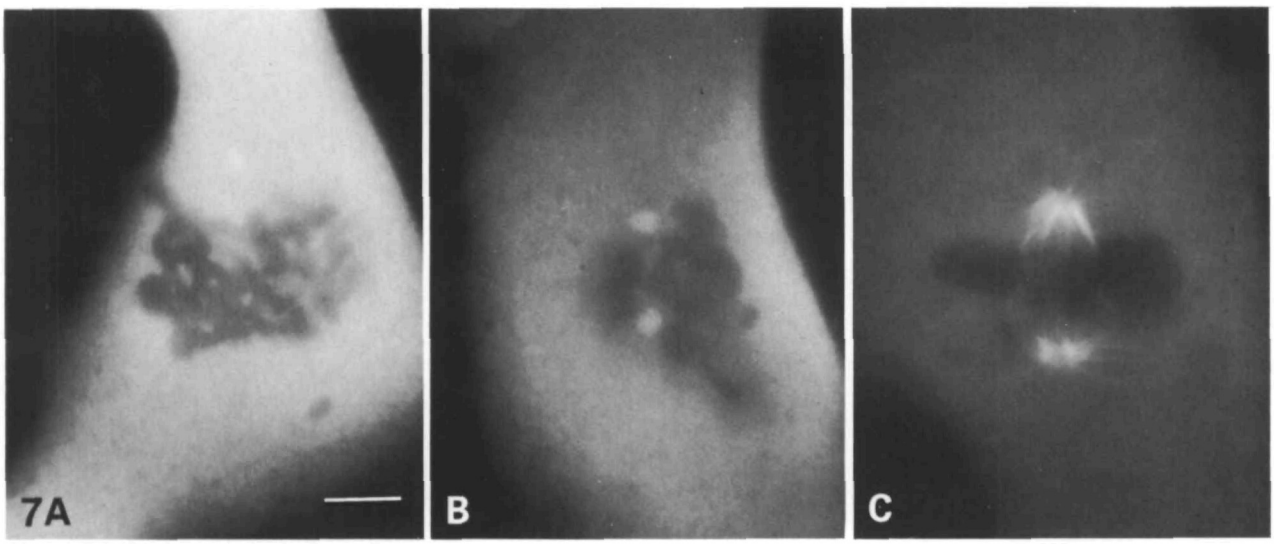

Fig. 7. Stabilization of microtubules against 2·5 μM-nocodazole by Co^{2+} in mitotic PtK1 cells. A. Nocodazole for 10 min; B, 1 min exposure to $CoCl_2$ followed by 10 min exposure to nocodazole plus $CoCl_2$; C, 1 min exposure to $CoCl_2$, 10 min exposure to chloral hydrate plus $CoCl_2$, brief rinse, and then 10 min in nocodazole.

these cells, some microtubules were preserved (Fig. 7C). A summary of the effect on microtubules of the various drug treatments is given in Table 2.

Effect of chloral hydrate on interphase cells

Exposure to chloral hydrate for up to 10 min did not affect interphase cell microtubules as determined by immunofluorescence (not shown), but the nuclear envelope and nucleolus became very refractile as seen by phase-contrast microscopy.

General observations

In both metaphase and interphase cells treated with 0·1 % chloral hydrate applied extracellularly, the structure of the mitochondria became altered: they appeared as small dots instead of as the usual thin lines seen by phase-contrast microscopy (100× objective, numerical aperture of 1·3). Such alterations in structure are frequently seen in mitochondria damaged by elevated levels of calcium (Carafoli, 1982). Observations of living cells showed that saltatory motion continued in cells bathed in 0·1 % chloral hydrate.

Table 2. *Summary of the effects of chloral hydrate, $CoCl_2$ and nocodazole on microtubules in PtK1 cells*

	Mitotic microtubules	Interphase microtubules
Control	+++	+++
Chloral hydrate	+	+++
$CoCl_2$	+++	+++
$CoCl_2$ + chloral hydrate	++++	+++
$CoCl_2$, rinse, chloral hydrate	+	+++
Nocodazole	−−−	−
$CoCl_2$ + nocodazole	−−−	−
$CoCl_2$ + chloral hydrate, rinse, nocodazole	+	+

++++, More than normal number; +++, normal number; +, some; −, very few; −−−, none. Each treatment was for 10 min. Concentrations: 0·1 % chloral hydrate, 10 mM-$CoCl_2$, 2·5 μM-nocodazole.

Discussion

We found that addition of chloral hydrate to PtK cells caused the concentration of intracellular free Ca^{2+} to rise within 15 s of application, peaking within a minute at levels two to seven times greater than baseline. The Ca^{2+} concentration then slowly decreased, but had not returned to basal levels by 12 min. Using tubulin immunofluorescence, we found that a 2–5 min exposure to chloral hydrate yielded very short metaphase spindles with abnormally long aster microtubules. A 10 min exposure to chloral hydrate caused both metaphase and anaphase spindles to lose aster and interpolar microtubules, but interphase microtubules were unaffected. Both the spindle shortening and microtubule loss were prevented by treatment with $CoCl_2$. These results suggest that the chloral hydrate-induced shortening of metaphase spindles and the inhibition of anaphase spindle elongation by chloral hydrate are due to sustained high levels of Ca^{2+}. They also indicate that mitotic microtubules are more labile to Ca^{2+} than interphase microtubules.

The effect of elevated levels of intracellular Ca^{2+} on mitotic and interphase microtubules *in vivo* has been studied previously by either injecting $CaCl_2$ into the cell (Kiehart, 1981; Izant, 1983; Keith *et al.* 1983; Keith, 1987) or by exposing cells to A23187, a Ca^{2+} ionophore (Fuller & Brinkley, 1976; Huchon & Ozon, 1985; Keith *et al.* 1983). In none of the above studies was the difference in Ca^{2+} lability between interphase and mitotic microtubules compared directly, although Ca^{2+} concentration was a major factor in inducing microtubule breakdown. Injection of $CaCl_2$ produced rapid but transient breakdown of microtubules in the region of injection, as the cells quickly removed the excess free Ca^{2+} (Kiehart, 1981; Izant, 1983; Keith *et al.* 1983; Keith, 1987). A23187 produced generalized microtubule breakdown in interphase cells when the external Ca^{2+} concentration was sufficiently high (Fuller & Brinkley, 1976; Keith *et al.* 1983). Our interpretation that the effects of chloral hydrate on mitosis are *via* Ca^{2+} elevation fits well with a conclusion from the above studies, in that an increase in intracellular free Ca^{2+} is adequate to produce microtubule breakdown *in vivo*. In addition, the early pattern and timing of microtubule breakdown and spindle shortening in prometaphase PtK cells after injection of either $CaCl_2$ or Ca–calmodulin (Keith, 1987) is very similar to that observed in our chloral hydrate-treated metaphase cells. During the 30 s to 3 min post-injection time, both kinetochore and interpolar microtubules became shorter, while astral microtubules appeared normal (Keith, 1987). Chloral hydrate-treated cells showed a similar time course (see Fig. 4B,C).

Our results with chloral hydrate are also in agreement with earlier work showing that mitotic microtubules are more labile than interphase microtubules (DeBrabander *et al.* 1986). Our data extend these findings, in that we have found that mitotic microtubules appear to be more Ca^{2+}-labile than interphase microtubules. This needs further work to be certain, since chloral hydrate-treated mitotic cells tended to have high levels of calcium for a slightly longer time than interphase cells (Table 1). Nonetheless, the observation of increased Ca^{2+} lability of mitotic microtubules is in accord with previous reports. For instance, Saxton *et al.* (1984) found that in PtK cells most mitotic microtubules turn over more rapidly than interphase microtubules. However, a simple model in which Ca^{2+} prevents assembly of newly polymerizing microtubules cannot explain the difference in Ca^{2+} lability, since the half-time for turnover of mitotic microtubules is only 20 s, whereas it is 3–5 min for interphase microtubules as determined by FRAP analysis (Saxton *et al.* 1984). Depolymerization of mitotic microtubules in cells exposed to chloral hydrate took at least 7 min, which is considerably longer than the time required for the complete turnover of most spindle microtubules. Furthermore, recovery of metaphase spindle structure after removal of chloral hydrate took longer than 2 min (data not shown). Therefore, the difference in Ca^{2+} lability of interphase and metaphase microtubules is probably due to the interaction of Ca^{2+} with another factor rather than to Ca^{2+} acting directly on microtubules.

The prevention by $CoCl_2$ of chloral hydrate-induced spindle shortening and microtubule breakdown could occur by two mechanisms. (1) Cobalt ions blocking the influx of Ca^{2+}, as has been shown for muscle cells (Kohlhardt *et al.* 1973); or (2) cobalt ions entering the cell and binding to microtubules, stabilizing them against Ca^{2+}-induced breakdown. Co^{2+} has been shown to protect microtubules *in vitro* against Ca^{2+}

and colchicine (Wallin *et al.* 1977). In our study, Co^{2+} does not enter normal PtK cells in sufficient quantity to stabilize mitotic microtubules against nocodazole. However, when cells were treated with chloral hydrate in the presence of $CoCl_2$, Co^{2+} entered the cell. This is based on three observations. First, when attempting to measure Ca^{2+} in Fura-2-loaded cells treated with $CoCl_2$ and chloral hydrate, the basal levels were stable and normal in the presence of Co^{2+}, but the fluorescence faded at both wavelengths 1–2 min after adding chloral hydrate. It was determined that Co^{2+} quenches Fura-2 fluorescence *in vitro* (unpublished observations). Second, after treatment with $CoCl_2$ and chloral hydrate, followed by nocodazole, some mitotic microtubules remained (Fig. 7C). Third, the metaphase spindle is larger, with longer asters, in cells treated with $CoCl_2$ and chloral hydrate (Fig. 6C).

The influx of Co^{2+} induced by chloral hydrate suggests that chloral hydrate is acting on the plasma membrane. There are additional reasons for thinking that the rise in intracellular free Ca^{2+} produced by chloral hydrate involves an interaction with one or more sites on the plasma membrane. One site of chloral hydrate action is the plasma membrane Ca^{2+}/Mg^{2+}-ATPase (Bergesse *et al.* 1983). Other membrane ATPases are also inhibited by chloral hydrate although they are less sensitive than the Ca^{2+}/Mg^{2+}-ATPase (Bergesse *et al.* 1983). Our finding that spindle elongation was inhibited when 0·1 % chloral hydrate was applied extracellularly, but not when 7·5 % was injected into the cell, also indicates that chloral hydrate effects are due to an interaction with the plasma membrane, rather than an intracellular site. In addition, in preliminary experiments, we found that 5 μM-TMB-8, a drug that inhibits Ca^{2+} release from the endoplasmic reticulum (Malagodi & Chiou, 1974), did not prevent the rise in Ca^{2+} levels induced by chloral hydrate. On the other hand, 10 mM-EGTA prevented or greatly reduced the Ca^{2+} response produced by chloral hydrate. These preliminary findings indicate that the rise in Ca^{2+} levels caused by chloral hydrate is due to Ca^{2+} entering the cell and not to Ca^{2+} being released from intracellular stores.

There is a possibility that the elevated Ca^{2+} levels have reduced the cell's ATP levels by damaging the mitochondria and thus is not affecting mitosis directly. For example, mitosis completely stops in *Xenopus* oocytes in which the ATP levels have been depleted to 50 % of normal by carbon monoxide (Epel, 1963). In dinitrophenol/deoxyglucose-treated cells, which have ATP levels reduced to 31 % of normal, saltatory motion and all anaphase movement stops (Spurck *et al.* 1986), but in chloral hydrate-treated cells saltatory motion, chromosome-to-pole movement and cytokinesis continued normally. So it is unlikely that chloral hydrate-treated cells have greatly reduced levels of ATP. It should be noted that the Ca^{2+} concentration in the above ATP-depleted cells (Epel, 1963; Spurck *et al.* 1986) was not determined, but a lack of ATP would certainly inhibit the Ca^{2+}/Mg^{2+}-ATPase.

In summary, we have presented evidence that chloral hydrate increases intracellular Ca^{2+} levels. The findings, that this rise occurs consistently, that elevations in Ca^{2+} have previously been shown to alter mitotic spindles in a similar way, and that Co^{2+} prevents these alterations, argue for elevated Ca^{2+} being the means by which chloral hydrate produces its effects on mitosis. We have not ruled out the possibility that chloral hydrate has other effects on the cell in addition to elevating Ca^{2+}. However, no other alteration in cell physiology is known to produce the pattern of microtubule breakdown that we have observed.

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