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Reduction of ultraviolet-induced mitotic delay by caffeine in G2-phase irradiated plasmodia of *Physarum polycephalum*

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Abstract. Synchronously mitotic surface Plasmodia of *Physarum polycephalum* were ultraviolet-irradiated at different times during G2-phase (– 4 h to – 20 min with respect to metaphase), and treated immediately thereafter with varying concentrations of caffeine. It was observed that ultraviolet-induced mitotic delay is reduced significantly by this methylxanthine. In plasmodia irradiated between – 4 and – 1 h with respect to metaphase, the effect was concentration-dependent and the need for a certain threshold dose for obtaining the reduction in delay was apparent. However, higher doses than this were fairly toxic when applied at this part of the cycle and led to more mitotic delay than that obtained with UV alone. The most striking observation made during this study was the phase-specific precipitous effect seen in those plasmodia irradiated at about 20 min before mitosis which almost eliminated the long delay due to ultraviolet-irradiation. These results are discussed in the context of some of the known effects of ultraviolet and caffeine on a mitosis-promoting factor. It is proposed that the significant reduction of ultraviolet-induced mitotic delay reported here is due to the reactivation of the ultraviolet-inactivated mitosis-promoting factor by caffeine. Alternatively, it is possible that caffeine may prevent the inactivation of this factor by ultraviolet.

Keywords. Ultraviolet-irradiation; caffeine; mitotic delay.

1. Introduction

Mitosis is known to get delayed for several hours by UV-irradiation in the synchronously mitotic plasmodia of *Physarum polycephalum* (Devi *et al* 1968). The response to UV (700 and 1400 Jm^{-2}) in this system is biphasic (Kumari and Nair 1984). The S-phase comprising the early one third of the cycle (Gl being absent) and the late G2-phase, including the UV-reversible, early part of prophase, forming the last 0·1 part of the cycle, are the two phases with extra sensitivity. Between these two, the S-phase is the more sensitive, as indicated by the duration of the UV-induced mitotic delay. However, the extra sensitivity seen during G2 is most relevant from the point of view of the mitotic process itself. Also, of particular interest in the present context is the condensation-decondensation cycle of the chromosomes during their traverse through the cell cycle, which itself is dependent on the level of histone phosphorylation. In *Physarum,* maximum phosphorylation of all histone types occurs during the transition from G2 to prophase, leading to condensation of chromosomes (Bradbury *et al* 1974; Brewer and Oleinick 1980), and inhibition of histone phosphorylation by perturbers such as γ-radiation was

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Abbreviations used: SDM, Semi-defined medium; PFM, post-fusion mitosis; MPF, mitosis promoting factor; MPA, maturation promoting activity.

shown to induce mitotic delay in this organism (Brewer and Oleinick 1980). In addition to causing delay, UV-irradiation is also known to reverse early prophase in *Physarum* (Devi *et al* 1968). We report here the reduction/elimination of UV-induced, G2-phase mitotic delay by different concentrations of caffeine in this organism. The concentration-dependency of this effect is easily demonstrable during much of the G2-phase. However, any concentration from 50 to 750 μg ml^{-1} was found sufficient to achieve this in plasmodia irradiated at about 20 min before mitosis. This is a time when early indications of chromosome condensation are visible under a microscope and extra sensitivity to UV is aslo seen (Devi *et al* 1968; Kumari and Nair 1984).

2. Materials and methods

Macroplasmodia (surface plasmodia) containing synchronously-dividing nuclei were prepared (Guttes and Guttes 1964) from shaken cultures of microplasmodia of *P. polycephalum* (M_3C strain) maintained on the semi-defined medium (SDM) of Daniel and Baldwin (1964). Both macro- and micro-plasmodia were maintained at 24°C. For irradiations a Philips 15-W germicidal lamp which is known to emit approximately 90% of the UV-energy at 2537Å (Jagger 1967) was used. The total dose delivered was 1400 Jm^{-2}, at a dose rate of 7·18 Jm^{-2} S^{-1}.

Aliquots from a stock solution (6 mg ml^{-1}) of caffeine (Sigma) made in SDM were added to the culture medium of the respective surface cultures, to get the different concentrations (50-750 μg ml^{-1}) of the drug employed here.

The sister macroplasmodia used in a set of experiments were prepared from pooled microplasmodial suspension. The perturbations were carried out at different times during the G2-phase preceding the second post-fusion mitosis (PFM). Each Plasmodium was cut into seven sectors. Six of them were irradiated and one served as control. Immediately after irradiation, five of them were transferred to SDM containing the respective concentration of caffeine and from then on the treatment was continuous. The mitotic delay in each category was estimated with respect to the untreated sector, taking metaphase as a reference point. The timing of mitosis in each sector was determined by phase-microscopic observation of ethanol-fixed smears (Guttes *et al* 1961). For comparison, sectors from a set of unirradiated Plasmodia were also treated for corresponding periods with different concentrations of caffeine.

3. Results

The present studies show that there are two aspects to the mitotic-delay reducing effect of caffeine on the UV-irradiated system. One is the concentration-dependency of the effect and the other its phase-specificity. What is perhaps most interesting is that the phase-specific effect was evident even with the lowest concentration of the drug employed by us.

Concentration-wise, mitotic delay was reduced most by 250 μg ml^{-1} of caffeine when irradiated at any time between –4 and –1 h before mitosis ('0' point being metaphase which lasts only for about 5 min), followed by 100 and 50 μg ml^{-1} in that order (table 1, early G2 experiments). This shows the requirement for a certain

Table 1. Effect of different concentrations of caffeine on UV-induced mitotic delay.

Category of experiment	Early G2			Late G2			Beginning of prophase	
Plasmodia	A	B	C	D	E	F	G	H
Time of UV-irradiation (h^{min}) before metaphase	3^{45}	2^{20}	1^{25}	0^{55}	0^{50}	0^{45}	0^{20}	0^{20}
Delay (h^{min})								
UV	9^{00}	8^{55}	7^{50}	9^{55}	7^{10}	9^{15}	9^{35}	11^{00}
UV + caffeine ($\mu g\ ml^{-1}$)	8^{25}	7^{55}	6^{15}	9^{30}	7^{15}	8^{55}	1^{20}	1^{10}
50	(6)	(11)	(20)	(4)	(101)*	(4)	(86)	(89)
100	9^{30}	6^{45}	4^{50}	9^{30}	1^{30}	9^{30}	1^{17}	1^{10}
	(106)*	(24)	(38)	(4)	(79)	(103)*	(87)	(89)
250	7^{10}	6^{10}	4^{05}	1^{40}	1^{05}	1^{20}	0^{50}	1^{00}
	(20)	(31)	(48)	(83)	(85)	(86)	(91)	(91)
500	8^{30}	9^{00}	9^{15}	1^{25}	1^{05}	1^{10}	0^{50}	0^{55}
	(6)	(101)*	(118)*	(86)	(85)	(87)	(91)	(92)
750	12^{00}	11^{55}	12^{35}	0^{45}	1^{25}	1^{25}	0^{50}	0^{45}
	(133)*	(134)*	(161)*	(92)	(80)	(85)	(91)	(93)

The data from 8 Plasmodia (A–H) are horizontally arranged, from the earliest to the latest, with respect to the perturbation timings in them, which is given with respect to the metaphase of the second PFM in the respective control sector. Plasmodia A–C, E and G are sisters from one experiment and F and H are sisters from another experiment. Plasmodium D is from a third experiment. The delays (calculated on the basis of the time of metaphase in the respective control sector) obtained in the different treated sectors of a Plasmodium are given sequentially below it (vertical column) and the percentage reduction/increase (*) in delay is given in parentheses. The percentage reduction/increase in delay has been calculated in the case of the different irradiated and caffeine-treated (UV + caffeine) sectors, taking the delay in the corresponding irradiated (UV) sector as 100%.

threshold dose for achieving the reduction in mitotic delay. However, at still higher concentrations, the mitotic delay reducing effect was superseded by the toxic effect of caffeine. For instance, 500 μg ml^{-1} of caffeine did not reduce mitotic delay when applied on plasmodia irradiated during this time (table 1, one out of three Plasmodia showed a small reduction) and with 750 μg ml^{-1}, the delay was even significantly more than that induced by UV alone. This probably may not be surprising, as considerable mitotic delay was induced in those plasmodial sectors which were exposed to 750 μg ml^{-1} of caffeine alone, starting –4 and –2 h respectively before metaphase (figure 1).

The phase-specific effect which was seen in plasmodia irradiated at about 20 min before metaphase is more striking, in that it is observed even with the lowest concentration (50 μg ml^{-1}) of the drug employed by us. In fact all the doses [50 μg ml^{-1} (0·26 mM) to 750 μg ml^{-1} (3·86 mM)] were highly effective at this time. These findings show that during transition from late G2 to prophase, there is a reduction in the threshold concentration requirements. A phase-specific effect was also seen in those plasmodia irradiated at any time between –1 h and – 40 min with respect to metaphase (table 1: late G2) and treated with the three higher concentrations (250–750 μg ml^{-1}) of the drug. Just as in those irradiated at the

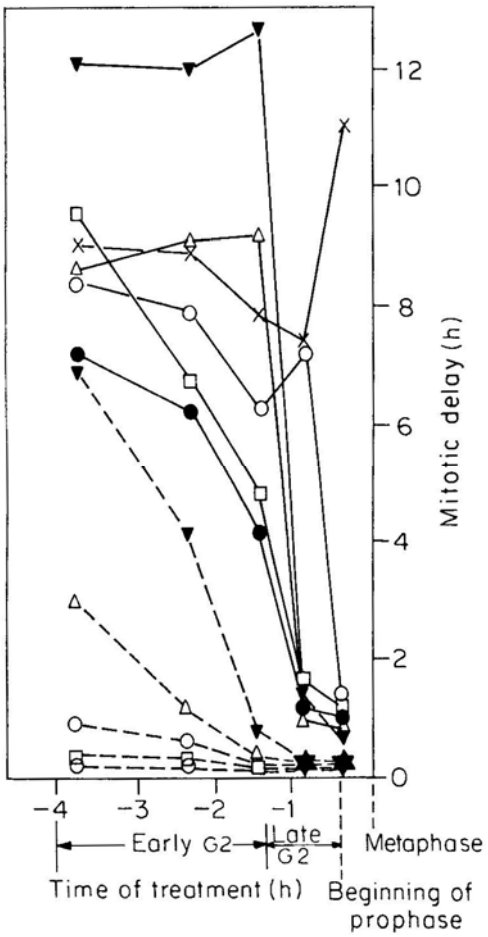

Figure 1. Effect of caffeine on UV-induced mitotic delay. Irradiation (1400 Jm^{-2}) was carried out at different times during the G2-phase preceding the second PFM and the sectors to be treated with caffeine were transferred to the drug containing medium (50–750 μg ml^{-1}) , immediately after irradiation and continuously treated till the delayed mitosis. (—), Irradiated plasmodia; (– – –), unirradiated plasmodia. Abscissa: Time (h) of irradiation and starting of caffeine treatment with respect to control metaphase, which served as the '0' point. The different cell cycle-phases are also marked on this axis. Ordinate: delay (h) due to the different perturbations, UV alone, UV and caffeine and caffeine alone respectively. (×), UV alone; (O), 50 μg ml^{-1}; (□), l00 μgml^{-1}; (●), 250 μg ml^{-1}; (Δ), 500 μg ml^{-1}; (▼),750 μg ml^{-1} of caffeine respectively. («) equivalent delay obtained with the different concentrations of the drug.

beginning of prophase, here also the mitotic delay reducing effect of the higher concentrations of the drug, rather than the toxic effect is seen. With the lowest concentration (50 μg ml^{-1}), the reduction when observed was only marginal and with 100 μg ml^{-1}, while significant reduction was observed in some plasmodia, others showed no reduction at all in experiments carried out during this period. Representative types are given in table 1.

4. Discussion

Oleinick *et al* (1978) reported a concentration-dependent reduction of γ-ray induced mitotic delay by caffeine in *P. polycephalum*. Their data show that, just as in the present studies, in those plasmodia irradiated earlier during G2 (74 min when compared to 27 min before control metaphase), the protective effect of the highest concentration of the drug employed by them (10 mM) was not evident because of the toxic effect on longer exposure.

The phase-specific effect reported by us is highly precipitous in nature and is temporally most interesting from the point of view of mitosis-controlling mechanisms. For example, this is the phase when maximal phosphorylation of H1 occurs in *P. polycephalum* (Bradbury *et al* 1974). The importance of phosphorylation of proteins involved in mitosis, particularly that of histones, in the initiation of mitosis and cell proliferation has been highlighted in a series of recent reviews (Dunphy and Newport 1988; Murray and Krischner 1989; Pardee 1989). It has been suggested by Daniel and Oleinick (1984) that the reduction in γ-ray induced mitotic delay by caffeine and some other phosphodiesterase inhibitors (Oleinick *et al* 1978) is due to adjustments in cyclic nucleotide levels. The change they observed in this case was a transient elevation of cyclic AMP followed by an increase in cyclic GMP. Earlier to this, some others have proposed the involvement of cyclic AMP in the reduction of X-ray induced mitotic delay in the case of mammalian cells (Scaife 1971; Boynton *et al* 1974; Walters *et al* 1974).

Mitosis-promoting factor (MPF) or the maturation-promoting factor, first reported to be present in the unfertilized egg cytoplasm of certain amphibians (Masui and Markert 1971; Reynhout and Smith 1974), has been ascribed with the function of phosphorylating some of the proteins involved in the structural changes associated with mitosis, such as chromatin condensation, nuclear envelope breakdown, etc (Murray and Krischner 1989). This protein has been assayed biochemically as histone H1 kinase or as a MPF in biological assays (O'Farrell *et al* 1989) and is in most respects similar to a certain maturation promoting activity (MPA), reported from various mammalian cells (Adlakha *et al* 1984). In another study, Adlakha *et al* (1988) have also reported a similar factor in extracts of *P. polycephalum* plasmodia, prepared 10–20 min before metaphase. On microinjection, it was found to induce germinal vesicle breakdown in oocytes of *X. laevis*.

The above finding by Adlakha *et al* (1988) is particularly interesting in the present context because of two reasons. Firstly, it is known from earlier studies that histone phosphorylation is critical for chromosome condensation and is an important metabolic step altered in *Physarum* during γ-rays induced mitotic delay (Brewer and Oleinick 1980). Secondly, a correlation is seen between the time when MPF activity is present in *Physarum* extracts (Adlakha *et al* 1988) and the time (approximately 20 min before metaphase) when caffeine has the maximum mitotic delay-reducing effect. Also, the transition point (a point beyond which the specific perturber has no effect) with respect to both γ-rays (Oleinick 1972) and UV (Devi *et al* 1968) lies at around this time in the cell cycle of *Physarum*. This is also a point where the plasmodia show extra UV-sensitivity (Kumari and Nair 1984). Adlakha *et al* (1984) find that the UV-induced decondensation of metaphase chromosomes is causally related to the loss of MPA in HeLa cells. They suggested that UV activates inhibitors of mitotic factors (IMF) in mitotic cells. These factors then bind

to MPF (called as MPA by them in this paper) to form an inert complex. Moreover, they observed that agents which promote chromatin condensation such as Mg^{2+} or polyamines partially restore the MPF of the UV-irradiated mitotic cell extracts.

Unlike UV, caffeine is known to induce premature chromosome condensation and advance mitosis in Syrian hamster fibroblast cells, depending on the DNA replicative state of the cell (Schlegel and Pardee 1986; Schlegel *et al* 1987). The chromosome-condensing effect of caffeine has also been observed in the case of *Physarum* (P R Jayasree and R Vimala Nair, unpublished results). While analysing the results of their study Schlegel and coworkers have suggested that the mitosis-advancing effect of caffeine is because of its ability to selectively stabilize a mitosis-related protein, which is generally fairly labile. Although this protein has not been characterized to any great extent by them, because of its mitosis-promoting activity and because of its G2-specificity, it would appear to be atleast related to the MPF (MPA) extracted from various cell types (Masui and Markert 1971; Reynhout and Smith 1974; Adlakha *et al* 1984; O'Farrell *et al* 1989) including *Physarum* (Adlakha *et al* 1988). It would then appear that the reduction of UV-induced mitotic delay in *Physarum,* reported here, could either be due to the reactivation of UV-inactivated MPF-like activity by caffeine or because of the prevention of its UV-inactivation by the drug. Further studies are in progress to elucidate this aspect of caffeine action.

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References

Adlakha R C, Wang Y C, Wright D A, Sahasrabuddhe C G, Bigo H and Rao P N 1984 Inactivation of mitotic factors by ultraviolet irradiation of HeLa cells in mitosis; *J. Cell Sci.* **65** 279-295

Adlakha R C, Shipley G L, Zhao J, Jones K B, Wright D A, Rao P N and Sauer H W 1988 Amphibian oocyte maturation induced by extracts of *Physarum polycephalum* in mitosis; *J. Cell Biol.* **106** 1445-1452

Boynton A L, Evans T C and Crouse D A 1974 Effects of caffeine on radiation-induced mitotic inhibition in S-180 ascites tumor cells; *Radiat. Res.* **60** 89-97

Bradbury E M, Inglis R J and Mathews H R 1974 Control of cell division by very lysine rich histone (Fl) phosphorylation; *Nature* (*London*) **247** 257-261

Brewer E N and Oleinick N L 1980 Histone phosphorylation during radiation-induced mitotic delay in *synchronous plasmodia Physarum polycephalum*; *Int. J. Radiat. of Biol.* **38** 697-702

Daniel J W and Baldwin H H 1964 Methods of culture for plasmodial myxomycetes; *Methods Cell Physiol.* **19**–41

Daniel J W and Oleinick N L 1984 Cyclic nucleotide responses and radiation-induced mitotic delay in *Physarum polycephalum; Radiat.Res.* **97** 341–351

Devi V R, Guttes E and Guttes S 1968 Effects of ultraviolet light on mitosis in *Physarum polycephalum*; *Exp. Cell Res.* **50** 589–598

Dunphy W G and Newport J W 1988 Unravelling of mitotic control mechanisms; *Cell* **55** 925-928

Guttes E, Guttes S and Rusch H P 1961 Morphological observations on growth and differentiation of *Physarum polycephalum* grown in pure culture; *Dev. Biol.* **3** 588-614

Guttes E and Guttes S 1964 Mitotic synchrony in the plasmodia of *Physarum polycephalum* and mitotic synchronisation by coalescence of microplasmodia; *Methods Cell Physiol.* **1** 43-54

Jagger J 1967 *Introduction to research in ultraviolet photobiology* (New Jersey: Prentice Hall)

Kumari P A V and Nair V R 1984 Mitotic delays and macromolecular synthesis in G2 phase-irradiated Plasmodia of *Physarum polycephalum*; *Exp. Cell Res.* **151** 104–111

Masui Y and Markert C 1971 Cytoplasmic control of nuclear behaviour during meiotic maturation of frog oocytes; *J. Exp. Zool.* **177** 129–146

Murray A W and Krischner M W 1989 Dominoes and Clocks: The union of two views of the cell cycle; *Science* **246** 614-621

O'Farrell P H, Edgar B A, Lakich D and Lehner C F 1989 Directing cell division during development; *Science* **246** 635-640

Oleinick N L 1972 The radiation-sensitivity of mitosis and the synthesis of thymidine kinase in *Physarum polycephalum*: a comparison to the sensitivity to actinomycin D and cycloheximide; *Radiat. Res.* **51** 638-653

Oleinick N L, Brewer E N and Rustad R C 1978 The reduction of radiation-induced mitotic delay by caffeine: a test of the cyclic AMP hypothesis; *Int. J. Radiat. Biol.* **33** 69-73

Pardee A 1989 Gl events and regulation of cell proliferation; *Science* **246** 603-608

Reynhout J K and Smith L D 1974 Studies on the appearance and nature of a maturation-inducing factor in the cytoplasm of amphibian oocytes exposed to progesterone; *Dev. Biol.* **38** 394-400

Scaife J F 1971 Cyclic 3',5'-adenosine monophosphate: its possible role in mammalian cell mitosis and radiation induced mitotic G2 delay; *Int. J. Radiat. Biol.* **19** 191-195

Schlegel R and Pardee A B 1986 Caffeine-induced uncoupling of mitosis from the completion of DNA replication in mammalian cells; *Science* **232** 1264-1266

Schlegel R, Croy R G and Pardee A B 1987 Exposure to caffeine and suppression of DNA replication combine to stabilize the proteins and RNA required for premature mitotic events; *J. Cell Physiol.* **131** 85-91

Walters R A, Gurley L R and Tobey R A 1974 Effects of caffeine on radiation-induced phenomena associated with cell-cycle traverse of mammalian cells; *Biophys. J.* **14** 99-118