Effects of Caffeine on Cleavage Delay of Sea Urchin Eggs
Induced by Ethidium Bromide or Puromycin

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The mitotic delay was induced when sea urchin eggs were treated with either ethidium bromide or puromycin, as observed with eggs fertilized with X- or UV-irradiated sperm. Treatment of these eggs with caffeine during the period of early prophase block resulted in the reduction of the mitotic delay. Protein synthesis of these eggs was not affected by X-irradiation but inhibited by ethidium bromide or puromycin. Caffeine was almost ineffective in changing the protein synthesis of eggs inseminated with X-irradiated sperm or treated with ethidium bromide. These facts mean that additive synthesis of protein is not required for the reduction by caffeine of the mitotic delay. Some role of protein synthesis in the reduction by caffeine of the cleavage delay is not excluded for puromycin treated eggs, since caffeine counteracted the inhibitory effect of puromycin on protein synthesis.

INTRODUCTION

Since the classical works of Henshaw or Yamashita et al., it is well established in sea urchin eggs that X-ray-irradiation induces the block at early prophase which is called “streak” stage.¹⁻⁴ However, the causes of division delay by irradiation have not been identified. In the previous papers, it was demonstrated that caffeine released X-ray-induced streak stage block only when the chemical was administered during the period of prolongation.⁵⁻⁶ The similar release from X-ray-induced G₂ block is shown in T-cells,⁷ Chinese hamster cells⁸ and S-180 ascites tumor cells.⁹ Metabolic inhibitors such as puromycin, a potent inhibitor of protein synthesis, and ethidium bromide, an inhibitor of deoxyribonucleic acid synthesis, are known to induce prolongation of the streak stage.¹⁰⁻¹² To examine caffeine effect on the mitotic delay induced by these inhibitors might be useful for elucidating the mechanism of mitotic delay by irradiation, and caffeine effect. The effects of caffeine on the mitotic delay induced by these inhibitors were, therefore, studied in the present experiment. Caffeine was found to reduce the length of the mitotic delay. Reduction occurred only when caffeine was administered during the streak stage.

MATERIALS AND METHODS

Gamete preparation. Gametes of the sea urchin, Hemicentrotus pulcherrimus,
were obtained as described previously. Eggs fertilized with diluted sperm were developed at approx 20°C.

**X- and UV-irradiation.** X-rays at a dose rate of 500 R/min was administered to the "dry" sperm for 10 min. UV-light was delivered to the diluted sperm for 15 sec at a dose rate of 20 ergs/mm²/sec. In this case, eggs were cultured in the dark throughout the experiment to prevent photorecovery.

**Inhibitor treatment.** Ethidium bromide and puromycin were dissolved in sea water and added to the eggs 5 minutes prior to fertilization. Final concentration of each chemical was 50 μg/ml throughout the present experiment.

**Caffeine treatment.** Caffeine was dissolved in normal sea water in the experiment using UV-light or X-rays, or in sea water containing ethidium bromide or puromycin. Caffeine was added at various times after fertilization, and final concentration of caffeine was adjusted to 2 mM. Ten minutes later caffeine was removed by washing with fresh normal sea water in the case of UV-light experiment, or with fresh sea water containing ethidium bromide or puromycin. Control eggs were also washed with these fresh solution to maintain the similar conditions. The cleavage time of these eggs was defined as the time when 50 per cent eggs had cleavage furrows.

**Assay of protein synthesis of caffeine-treated eggs.** Eggs were cultured at a concentration of 4,500 to 8,000 eggs/ml. In this case, caffeine was added when the streak stage started and was not removed. ¹⁴C-leucine (312 mCi/mole), and sometimes ³H-leucine (32 Ci/m mole), streptomycin (50 μg/ml) and penicillin (100 U/ml) were added 5 minutes prior to fertilization. An 0.5 ml or 1 ml aliquot of eggs was taken into test tubes at various intervals from fertilization and fixed immediately by adding 5 ml of 5 per cent ice-cold trichloroacetic acid solution containing 25 mM unlabeled leucine. Procedure of washings with ice-cold and hot trichloroacetic acid solution followed that described by Fry and Gross. After one washing with cold methanol and two washings with methanol at room temperature, whole sample was dissolved in 0.5 ml of hyamine hydroxide. Radioactivity was counted by adding 10 ml of toluene scintillation cocktail. Counting efficiency was about 78-80 per cent for ¹⁴C-leucine or 24-26 per cent for ³H-leucine. All experiments were repeated three to four times with almost similar results.

**RESULTS**

When sea urchin eggs were fertilized with X-irradiated sperm, the prolongation of streak stage in the first cleavage was induced, and caffeine reduced the prolonged streak stage only when the chemical was administered during the period of the prolongation. Caffeine manifested exactly similar effects on mitotic delay induced by UV-irradiated sperm (Fig. 1). Caffeine achieved the effects only when administered during the prolonged streak stage.

Effects of caffeine on the cleavage delay induced by puromycin and ethidium bromide were, then, studied (Fig. 2). Treatment of puromycin or ethidium bromide
induced the prolongation of the streak stage which caused the cleavage delay. Pulse-treatment of caffeine during the period of prolonged streak stage was also effective in reducing the cleavage delay induced by these inhibitors, although caffeine further delayed the cleavage time when administered during the periods other than prolonged streak stage. It is of great interest that the exactly similar caffeine-sensitivity curve was observed with X-ray- or UV-induced mitotic delay of sea urchin eggs (Fig. 1 and (5)).

These metabolic inhibitors might produce a profound effect on protein synthesis of the sea urchin eggs. An attempt was, therefore, made to find a possible relationship between the caffeine effect and modification of the protein synthesis by caffeine (Fig. 3). Protein synthesis was determined by measuring incorporation of labeled leucine into acid insoluble fraction. Incorporation by normal eggs promptly increased after fertilization. Caffeine at 2 mM did not change normal incorporation when added at the beginning of the streak stage. X-irradiation of the sperm did not cause an inhibition of these normal protein synthesis (Fig. 3a). Ethidium bromide and puromycin partly inhibited total protein synthesis of normal eggs (Fig. 3b and 3c). Caffeine at 2 mM, a dose causing the reduction of cleavage delay
delay, hardly affected protein synthesis of X-irradiated or ethidium bromide treated eggs (Fig. 3 a and 3 b). On the other hand, puromycin-inhibited protein synthesis was recovered to some extent by adding caffeine (Fig. 3 c). It was confirmed in each case that delayed cleavage time was shortened by caffeine as indicated by arrows in the figure (Fig. 3). Puromycin with a concentration higher than 100 µg/ml and sometimes even 50 µg/ml completely inhibited the cleavage. Continuous treatment of such eggs with 2 mM caffeine sometimes induced cleavage after long time (100-300 min.) and recovered the inhibited protein synthesis. It seems probable from these results that the mitotic delay induced by X-ray and ethidium bromide, and its reduction by caffeine bear no definite relation to modification of protein synthesis. However, some role of protein synthesis in the caffeine effect is not excluded for puromycin treated eggs, since caffeine counteracted the inhibitory effect of puromycin on protein synthesis.

**DISCUSSION**

Puromycin and ethidium bromide are known to induce the arrest of the cell division at early prophase as observed with the eggs fertilized with X- or UV-irradiated sperm. In the present study, it was shown that the cleavage delay induced by ethidium bromide or puromycin was reduced by caffeine treatment. These reductions of cleavage delay occurred only when caffeine treatment was performed during the period of prolonged streak stage. The above agents, although having the same effect on the cleavage might have the mode of actions different from each other. Puromycin inhibits protein synthesis, and ethidium bromide interacts with deoxyribonucleic acid and inhibits its synthesis, although it remains unclear wheth-
er these actions really result in the cleavage delay. UV-induced cleavage delay might be due to thymine dimer on deoxyribonucleic acid molecules.\textsuperscript{14,15} X-ray-induced primary damage responsible for cleavage delay has not been clarified.\textsuperscript{16-18} In spite of these difference in mode of action, the eggs treated with metabolic inhibitors or the eggs fertilized with X- or UV-irradiated sperm were arrested at the same stage of the cleavage cycle, and this arrest was released by caffeine treatment. Then, it becomes a question whether caffeine acts in the same manner in all cases. Studies on the effect of caffeine on protein synthesis suppressed by puromycin and ethidium bromide (Fig. 3) indicate that caffeine affects in different manner on the inhibited synthesis. Nevertheless, it can be said that actions of caffeine are closely related to some biological events occurring at the streak stage, since caffeine treatment was effective only during the prolonged streak stage. 

Fig. 3. Effect of caffeine on labeled leucine incorporation into acid insoluble fraction of eggs inseminated with X-irradiated sperm (a), eggs treated with ethidium bromide (b) and eggs treated with puromycin (c). Radiochemicals used were $^1$C-leucine at a concentration of 0.2 pCi/ml (a) and 0.6 pCi/ml (c), and $^3$H-leucine at a concentration of 1 pCi/ml (b). In all the cases, incorporation of normal eggs was indicated by (○—○). Incorporation by eggs fertilized with X-irradiated sperm (5,000 R), by eggs treated with ethidium bromide (50 μg/ml) or by eggs treated with puromycin (50 μg/ml), was indicated by (●—●). 2 mM caffeine was added 45 (a), 47 (b) and 43 (c) minutes after fertilization which was the start point of streak stage and was not removed (△—△). The first cleavage time in each case was indicated by arrows. Normal eggs (●). Eggs fertilized with X-irradiated sperm or treated with ethidium bromide or puromycin (○). Eggs treated with caffeine (△). Egg number of each sample was 2,300 (a), 3,500 (b) and 8,000 (c).
According to Vacquir and Brachet, ethidium bromide inhibits the first step of chromatin aggregation. In addition, our preliminary experiment showed that mitomycin C, known as X-ray mimetic reagent which cuts deoxyribonucleic acid strands, induced the cleavage delay, and that this cleavage delay was also reduced by caffeine. These facts strongly suggest that the effect of caffeine might be related to chromatin aggregation or some damage(s) on deoxyribonucleic acid molecules. Caffeine was also effective in releasing the puromycin-induced arrest (Fig. 2), and recovered to some extent the protein synthesis inhibited by puromycin. Therefore, this recovered synthesis might contribute to the reduction of cleavage delay induced by puromycin. It has been suggested that some protein synthesis is necessary for the recovery from X-ray induced damage and the recovered protein synthesis might be partly related to these recovery from the damage. On the other hand, the additive protein synthesis was not required for the reduction by caffeine of the cleavage delay in the eggs fertilized with X-irradiated sperm and the eggs treated with ethidium bromide (Fig. 3). It was also shown in our preliminary experiment that caffeine reduced the cleavage delay induced by a high dose (800 μg/ml) of chloramphenicol, an inhibitor of protein synthesis, without additive protein synthesis.

There was another postulated mechanism interpreting the caffeine effect. As caffeine is known to increase intracellular c-AMP levels by inhibiting c-AMP phosphodiesterase activity, c-AMP is considered to be one of the probable candidates for the mediator of caffeine action on mitotic inhibition. There are both positive and negative data to this possibility as discussed in the previous paper. As it was shown that exogeneously added c-AMP passes through the membrane, the fact that X-ray-induced cleavage delay could not be shortened by exogeneously added c-AMP might exclude this possibility.

Furthermore, several other effects of caffeine, e.g. enhancement of X-ray-induced chromosomal aberration and induction of change in intracellular distribution of calcium are reported. However, the relation of these effects to the reduction of the cleavage delay is not clear.

To conclude so far as the present results concerned, it seems to be difficult to interpret the reduction by caffeine of the cleavage delay only in terms of changes in c-AMP levels or protein synthesis. Further experiments might be necessary.

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