Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 746-750

# POTENTIATION of K252a, A PROTEIN KINASE INHIBITOR-INDUCED POLYPLOIDIZATION,BY cAMP IN CULTURED FIBROSARCOMA CELL LINE

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Received October 17, 1994.

SUMMARY: We found that K252a, a potent inhibitor of protein kinases (PK), induced DNA re-replication of Meth-A cells, i.e., DNA synthesis at a higher DNA ploidy without undergoing cytokinesis (polyploidization). The K252a-induced polyploidization was inhibited by phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, suggesting that the polyploidization is caused through inhibition of PKC. By contrast, the polyploidization was potentiated by adenosine 3':5'-cyclic monophosphate (cAMP), a cAMP-dependent protein kinase (PKA) activator. These findings suggest that the cAMP-dependent signaling pathway and diacylglycerol (DAG)-dependent signaling pathway play an important role in regulating the induction of polyploidization in Meth-A cells, through a possible "cross-talk" between the two pathways. 

K252a is a member of a group of natural alkaloids (staurosporine, K252b) present in the culture broth of Nocardiopsis sp. and reported to exert biological activity, inhibiting PK such as the PKC and PKA, by competing with the binding of ATP to the kinase catalytic domain (1-2). The phosphorylation of proteins plays a key role in the regulation of cellular functions. Recently, there has been increasing evidence for the regulation of the eukaryotic cell cycle by a variety of PK (3-6). More recently, K252a was found to cause polyploidization without an intervening mitosis, characteristically inducing the formation of tetraploid cells with an 8C DNA content, suggesting that PK sensitive to K252a are involved in the blocking of polyploidization (7). Although significant progress has been made toward the elucidation of the mechanism of the polyploidization, the roles of the signal transduction pathway in regulating the induction of polyploidization remain obscure.

In most tissues, at least two signal transduction pathways for the control of cellular activities by various extracellular signals have been identified, each putatively associated with the activity of a distinct PK. One is related to a cAMP-dependent signaling pathway (CSP) which stimulates PKA (8,9), whereas the other is a DAG-dependent signaling pathway (DSP) which stimulates PKC (8,10). Much attention has been focused on the interaction between different second messenger pathways acting on various signaling systems (11).

0006-291X/94 $5.00

We examined the possible participation of the signal transduction pathways in regulating the induction of polyploidization and attempted to dissect the relationship between the CSP and DSP further. The findings suggest that CSP and DSP play an important role in regulating the induction of polyploidization and suggest that CSP and DSP participate in the regulation of the induction of polyploidization by means of cross-talk between the two pathways.

## Materials and methods

### Materials

K252a was purchased from Funakoshi Co., Japan, dissolved in dimethyl sulfoxide (DMSO) and stored in the dark at -20°C. $N^6$,2'-O-dibutyryl cAMP (DB-cAMP) and PMA were purchased from Sigma. DB-cAMP was dissolved in $H_2O$ and PMA in ethanol. All other chemicals were of reagent grade and purchased from Sigma.

### Cell line and culture

Meth-A, (MO, 3-methylcholanthrene-induced fibrosarcoma cell line, syngeneic to BALB/c mice) was grown in a mixture of Hams F-10 and L-15 (ratio, 3:7) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories), 50 units/ml of penicillin and 50 μg/ml of streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$. The viability of the tumor cells used in these experiments was consistently more than 95% when evaluated by the trypan blue exclusion method.

### Flow cytometric measurements

Flow cytometry was employed to determine the DNA content of Meth-A cells. After fixation in 70% ethanol, the cells were treated exhaustively with pancreatic RNase A and stained with propidium iodide (50 μg/ml in PBS). Fluorescence from individual cells was measured with a Cytofluorograf System 50H (Ortho Instruments) equipped with a 4W argon ion laser. The fluorescence of individual cells irradiated with a focused laser light at a wavelength of 488 nm (200 mW laser power) was detected by a photomultiplier tube. The relative intensities of red fluorescence were measured and DNA histograms were obtained.

## Results

### Induction of polyploidization by K252a

Exponentially growing cells were challenged with 1 μM of K252a and then cultivated for 0-36 hrs. Fig.1 shows the DNA histograms obtained from cultures of Meth-A cells treated with K252a. Immediately after exposure, the ratio of 8C (C=haploid DNA content) to 2C was 0.08. With continued incubation, cells with progressively greater DNA contents were observed until 36 hrs. At the 36-hour time point, the ratio of 8C to 2C was up to 0.45 and three sharp peaks corresponding to 2C, 4C and 8C were observed. Moreover the main peak shifted from 2C in the control to 4C.

### Potentiation of the polyploidization by DB-cAMP

Fig. 2 demonstrates the increase in DNA content following treatment with 1 μM of K252a plus various concentrations of DB-cAMP. The polyploidization could markedly be up-regulated by as little as 3.125 μM of DB-cAMP. The main peak shifted from 4C of polyploidization to 8C, a remarkable population of cells with a 16C DNA content appeared, and the ratio of 16C to 2C was 0.82. At a concentration of 6.25 μM, an increase in the 8C cells was evident. Optimal potentiation of the polyploidization by DB-cAMP was observed at a concentration of 12.5 μM, ratio of 16C to 2C was up to 4.00 and the main peak shifted to 16C further.

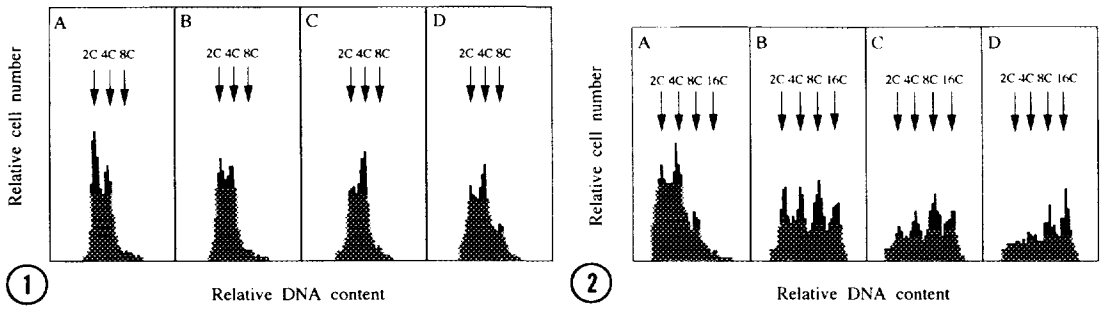


**Figure 1.** Induction of polyploidization by K252a. Meth-A cells were treated with 1 μM of K252a for 0 (A), 12 (B), 24 (C) and 36 hrs (D). The cells were collected and distributions of DNA content were determined by flow cytometry.

**Figure 2.** Potentiation of the polyploidization by DB-cAMP. Meth-A cells were treated with 1 μM of K252a (A) in the presence of 3.125 (B), 6.25 (C) and 12.5 μM (D) of DB-cAMP for 36 hrs. The distributions of DNA content in the treated cells were determined by flow cytometry.

## Down-regulation of the polyploidization by PMA and ATP

The relationship between the induction of the polyploidization and DSP was examined using PMA as a stimulant. As shown in table 1, PMA produced a marked and dose-dependent inhibition of the polyploidization. The percentage of cells with an >8C DNA content determined after 36 hrs of treatment with 1 μM of K252a in the presence of 0.01 μM PMA was found to decrease. At PMA concentrations exceeding 0.1 μM, the increase of cells with an >8C DNA content was completely reversed. Similar results were obtained when the Meth-A cells were treated with 1 μM of K252a in the presence of ATP. ATP at 100 μM almost completely abolished the polyploidization. These findings suggest the polyploidization through inhibition of PKC.

**Table 1. Down-regulation of the polyploidization by ATP and PMA**

| | Distribution of DNA content | | |
|---|---|---|---|
| | 2C | 4C | >8C |
| Control | 46.9 | 42.9 | 10.2 |
| K252a (1 μM) | 38.5 | 44.2 | 17.3 |
| K252a (1 μM) + PMA (0.01 μM) | 41.9 | 44.2 | 14.0 |
| K252a (1 μM) + PMA (0.1 μM) | 41.5 | 49.1 | 9.4 |
| K252a (1 μM) + PMA (1 μM) | 47.1 | 43.1 | 9.8 |
| K252a (1 μM) + ATP (10 μM) | 32.6 | 48.8 | 18.6 |
| K252a (1 μM) + ATP (100 μM) | 44.7 | 44.7 | 10.6 |

The distributions of DNA content in control Meth-A cells without K252a treatment, in Meth-A cells treated with K252a alone, and in Meth-A cells treated with K252a in the presence of ATP or PMA for 36 hrs were determined.

**Prevention of cycloheximide from DB-cAMP-mediated potentiation of the polyploidization**

To examine the possible requirement of *de novo* protein synthesis for the polyploidization and DB-cAMP-mediated potentiation, we examined whether cycloheximide (CHX), a protein synthesis inhibitor, affects the above-mentioned effects. As shown in Fig. 3, in the presence of K252a + DB-cAMP + CHX, the DNA distribution was similar to that seen in the presence of K252a alone, namely, although the height of the peak was higher than that seen in K252a addition alone, the main peak shifted from DB-cAMP plus K252a-induced 16C back to 4C and the peak corresponding to 16C disappeared. These findings indicate that *de novo* protein synthesis is required for the DB-cAMP-mediated potentiation, but not for the polyploidization.

## Discussion

In the present study, polyploidization of Meth-A cells was induced by the addition to the medium of a potent inhibitor of PKC, K252a, which has been reported to induce polyploidization of rat diploid fibroblasts (3Y1) (7). PKC is considered to play a pivotal role in the regulation of cell growth and proliferation through its activation by growth factors and other agonists (12-14). PKC is also believed to be the intracellular receptor for phorbol esters, which are tumor promoters that mimic the activating effect of DAG (15-17). We found that a phorbol ester, PMA reversed the polyploidization. This strongly suggests that DSP is involved in the down-regulation of polyploidization.

There are many studies which indicate that K252a also exerts an inhibitory effect on PKA (1-2). Hence it is not surprising that cAMP which plays a role through activation of PKA could produce an inhibitory effect on the polyploidization resembling PMA. However, interestingly we found that cAMP potentiated, but did not inhibit, the polyploidization. This implied that the induction of the polyploidization was not due to the inhibition of PKA. Similarly, the formation of dendrites by melanocytes has been reported to be induced by cAMP and further accelerated by staurosporine, an analogue of K252a (18). One explanation for the potentiating effect of cAMP on the polyploidization is that CSP inhibits DSP. In agreement with the above-mentioned finding, evidence from certain tissues, such as neutrophils, platelets, lymphocytes (19) and myoblasts (20),

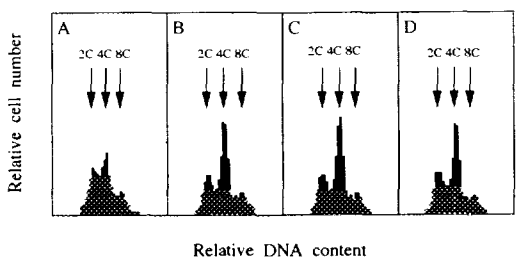


**Figure 3.** Prevention of cycloheximide from DB-cAMP-mediated potentiation of the polyploidization. The distributions of DNA content in the Meth-A cells treated with 1 μM of K252a (A) in the presence of 0.5 μM of cycloheximide along with 3.125 (B), 6.25 (C) and 12.5 μM (D) of DB-cAMP for 36 hrs were determined.

showed that CSP produces an inhibitory effect on DSP. The molecular mechanisms of "cross-talk" between the two pathways regarding the regulating polyploidization of Meth-A cells are under further investigation.

*De novo* protein synthesis is required for the accumulation of activin mRNA mediated by the CSP (21). We found that *de novo* protein synthesis is also required for the cAMP-mediated potentiating effect of the polyploidization. It is a logical extension to hypothesize that the cellular factor(s) involved in the process of cAMP-mediated potentiating effect are synthesized. If true, it is very important to identify the characters of the cellular factor(s).

In conclusion, our findings indicate that K252a induced polyploidization of Meth-A cells. PKC activator PMA suppressed the effect of the polyploidization, suggesting that it is caused through inhibition of PKC. cAMP potentiated the polyploidization and the cAMP-mediated potentiating effect was abolished by addition of CHX. One possible explanation is that cAMP-induced cellular factor(s) involved in the up-regulation of the polyploidization through a possible "cross talk" between CSP and DSP.

## Acknowledgment

This study was supported in part by a grant from the Science Research Promotion Fund (1994) of the Japan Private School Promotion Foundation, Japan.

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