Cell membrane changes of structure and function in protein kinase inhibitor-induced polyploid cells

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Abstract. Exogenous cyclic AMP has been thought to be a chemical without marked pharmacological effect until now, as it is not capable of penetrating the cell membrane in most eucaryotic cells. The present study obtained results consistent with those of most previous studies, showing that exogenous cyclic AMP itself did not interfere with the cell cycle even at the high dose of 100 μ M. However, it was found that K252a, a potent inhibitor of protein kinases including protein kinase C, induced DNA re-replication, i.e. DNA synthesis at a elevated DNA ploidy in cells that had not undergone cytokinesis (leading to polyploidization), and that exogenous cyclic AMP markedly potentiated the K252a-induced polyploidization at a very low dose similar to the effective dose of membrane-permeable cyclic AMP analogue dibutyryl cyclic AMP. These findings suggested that the cell membrane changed during the formation of polyploid cells. This supposition was confirmed by scanning electron microscopy to observe structural changes and by determination of cellular attachment to investigate functional changes.

INTRODUCTION

It is well known that the cell membrane enclosing every eukaryotic cell is more than a passive barrier. The lipid bilayer of the cell membrane is of major importance in determining many features of behaviour and mediating the various functions of the eukaryotic cell, in which it serves as a relatively impermeable barrier to the flow of most water-soluble molecules, transports specific molecules into or out of the cells, acts as a barrier to the entry of important information from the extracellular environment, and uses a variety of molecular mechanisms for transducing extracellular signals across the cell membrane.

Cyclic adenosine monophosphate (AMP) is a ubiquitous intracellular second messenger and, through activation of protein kinase A, plays important roles in the regulation of various cellular processes in a wide variety of organisms (Edwards *et al.* 1993, Hajnoczky *et al.* 1993, Ishizuka *et al.* 1994, Ouedraogo, Stoclet & Bucher 1994, Vandromme *et al.* 1994). In general, it is believed that exogenous cyclic AMP has no marked pharmacological action as it

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is not capable of penetrating the cell membrane in most eukaryotic cells (Posternak, Sutherland & Henion 1962, Schultz *et al.* 1994). The mechanism whereby cell membranes shut out exogenous cyclic AMP remains obscure.

Protein kinase C, a multi-gene family of enzymes consisting of at least 12 different serine-threonine kinases, translocates from the cytosol to the cell membrane and nuclear membrane in response to activators (Nishizuka 1986, Hartwig *et al.* 1992, Singh *et al.* 1994, Ikizawa *et al.* 1996, Murray & Fields 1997), is thought to play a pivotal role in the regulation of cell growth and proliferation through its activation by growth factors and other agonists (Ha & Exton 1993, Kobayashi *et al.* 1994). Protein kinase C-mediated phosphorylation is also believed to regulate membrane functions, including the sensitization of β 2-adrenergic receptor to further agonist stimulation (Sibley & Lefkowitz 1985), and ion conductance channel activities of Ca^{2+} (Wang *et al.* 1993), K^{+} (Chen & Yu 1994) and Cl^{-} (Berger, Travis & Welsh 1993).

K252a is a member of a group of natural (staurosporine, K252b) alkaloids present in the culture broth of *Nocardia* sp. that are thought to exert their biological activities, inhibiting protein kinases, including protein kinase C, by competing with the binding of ATP to the kinase catalytic domain (Ross *et al.* 1995). K252a was found to affect cell membrane Na^{+} – K^{+} – 2Cl^{-} co-transport (Klein, Perry & O'Neill 1993), cell polarity and locomotor activity (Zimmermann & Keller 1992).

To clarify the possible mechanisms whereby exogenous cyclic AMP cannot exert pharmacological action, the effects of K252a on the morphology and function of cell membrane were examined in the present study. K252a was found to induce DNA re-replication (polyploidization), i.e. DNA synthesis at high DNA ploidy in cells that had not undergone an intervening mitosis (cytokinesis). Exogenous cyclic AMP itself did not affect the cell cycle, but it markedly potentiated the K252a-induced polyploidization at a very low dose. Cells treated with K252a showed clear changes in the cell surface and an increase in cell-substratum attachment as compared to the control cells. These findings suggest that the inhibition of K252a-sensitive protein kinase(s) might be a result of changes in the structure and function of the cell membrane.

MATERIALS AND METHODS

Materials

K252a was purchased from Funakoshi Co., Tokyo, Japan, dissolved in dimethylsulphoxide and stored in the dark at -20°C . Cyclic AMP and dibutyryl cyclic AMP were purchased from Sigma (St Louis, MO, USA) and dissolved in water. All other chemicals were of reagent grade and purchased from Sigma.

Cell line and culture

Meth-A cells (3-methylcholanthrene-induced sarcoma cell line, syngeneic to BALB/c mice) were grown in a mixture of Hams F-10 and L-15 (ratio 3:7) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, OH, USA), 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin (Micallef *et al.* 1997). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The viability of the cells used in these experiments was consistently greater than 95% when evaluated by the trypan blue exclusion method.

Growth curve

The number of Meth-A cells was determined by using a Coulter Counter model ZM and Channelyser model 256 (Coulter Electronics, FL, USA). The analyser was calibrated using 9.61 μm styrene beads.

Flow cytometric measurement

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 (10 µg/ml in phosphate-buffered saline (PBS)). Fluorescence from individual cells was measured with a Cytofluorograf System 50H (Ortho Instruments, NJ, USA) equipped with a 4 W 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. Relative intensities of red fluorescence were measured and DNA histograms were obtained. The results were analysed using CELLQuest software (Becton-Dickinson, NJ, USA). For polyploidy analysis, DNA histograms were divided into compartments that approximated the 2C, 4C and 8C DNA contents by gating events to the 2C, 4C and 8C peaks. The frequency of cells in each DNA compartment was calculated by dividing the number of cells in the compartment by the total number of cells in the histogram. Cell doublets and debris were excluded by gating on FL2 width and FL2 area dot plots. All experiments were repeated at least twice.

Assay of cell adhesion

Meth-A cells were seeded onto 60 mm tissue culture Petri dishes and treated with 1 µM K252a. After the indicated times, the unattached cells were collected from the medium after gently shaking the culture dishes. The numbers of unattached and attached cells were counted using the Coulter Counter. The percentage of cells attached to the culture dishes was calculated by the formula: attached cell number/total cell number per culture dish × 100.

Analysis of morphological changes by scanning electron microscopy

For assessment of morphology by scanning electron microscopy, cells were collected, washed 3 times with PBS, and fixed immediately with 2% glutaraldehyde in PBS at 4°C for 2 h. The samples were washed three times with PBS, post-fixed in 4% OsO₄ in PBS at 4°C for 1 h and washed three times with PBS. A drop of the samples was placed on coverslips, dried at 37°C overnight, and coated with gold. Samples were then examined in a scanning electron microscope (Jeol JSM-840) at 15 kV and micrographs were taken on Fuji Neopan SS 120 roll film.

RESULTS

K252a inhibits proliferation of Meth-A cells

For this study, exponentially growing Meth-A cells in unsynchronized cultures were treated with 0, 0.10, 0.50 or 1.00 µM K252a and then cultivated for 36 h. Cells were counted 0, 12, 24 and 36 h after K252a addition. Figure 1 shows the proliferation curves of the cells as a function of time of K252a treatment. Inhibition of the proliferation of Meth-A cells by K252a was clearly observed in a dose-dependent manner during the 36-h period.

K252a induces polyploidization in Meth-A cells

The DNA content of Meth-A cells treated with 0 and 1.00 µM K252a for 36 h was determined by flow cytometry. The distributions of cells with 2C (C = haploid DNA content), 4C and 8C DNA content are shown in Table 1. During 0–12 h of treatment, no marked effect of K252a on the cell cycle was detected. After 12 h, the percentage of cells with 8C DNA content was found to increase. Treatment with 1.00 µM K252a for 36 h clearly induced polyploidization of Meth-A cells.

Figure 1. Inhibition of proliferation by K252a in Meth-A cells. Meth-A cells were treated with 0, 0.10, 0.50 or 1.00 μM K252a for 36 h. The number of cells was determined using a Coulter Counter.

Table 1 Time course of K252a-induced polyploidization

Time (h)	Distribution of DNA content		
	2C	4C	8C
0	50.6 $\pm$ 4.8	43.4 $\pm$ 3.9	6.0 $\pm$ 1.4
6	49.1 $\pm$ 3.1	44.1 $\pm$ 5.4	6.8 $\pm$ 2.8
12	47.6 $\pm$ 4.4	46.2 $\pm$ 3.7	6.2 $\pm$ 3.9
24	38.1 $\pm$ 2.7	51.4 $\pm$ 6.2	10.5 $\pm$ 2.5
36	36.4 $\pm$ 2.3	47.1 $\pm$ 5.1	16.5 $\pm$ 2.5

Meth-A cells were treated with 1.00 μM of K252a for 0, 6, 12, 24, and 36 h. The cells were collected and the distribution of DNA content were determined by flow cytometry. Values are mean $\pm$ SD.

Next, the dose dependence of the effect of K252a on the cell cycle was investigated. Cells were treated with 0, 0.05, 0.10, 0.20, 0.50 or 1.00 μM K252a for 36 h. As shown in Table 2, a dose-dependent induction of polyploidization by K252a was observed. A slight increase of cells with 8C DNA content was observed at 0.20 μM K252a. Treatment with 1.00 μM K252a resulted in the appearance of a markedly increased proportion of cells with 8C DNA content.

Table 2 Dose-dependent induction of polyploidization by K252a

Dose (μM)	Distribution of DNA content		
	2C	4C	8C
0	50.6 $\pm$ 4.8	43.4 $\pm$ 3.9	6.0 $\pm$ 1.4
0.05	49.1 $\pm$ 5.3	44.1 $\pm$ 2.2	6.8 $\pm$ 3.1
0.10	49.6 $\pm$ 3.8	44.2 $\pm$ 2.2	6.2 $\pm$ 2.9
0.20	50.2 $\pm$ 4.1	42.3 $\pm$ 3.4	7.5 $\pm$ 2.3
0.50	42.2 $\pm$ 3.5	48.3 $\pm$ 3.7	9.5 $\pm$ 1.8
1.00	36.4 $\pm$ 2.3	47.1 $\pm$ 5.1	16.5 $\pm$ 3.7

Meth-A cells were treated with 0, 0.05, 0.10, 0.20, 0.50 or 1.00 μM K252a for 36 h. The cells were collected and the distributions of DNA content determined by flow cytometry. Values are mean $\pm$ SD.

Effects of exogenous cyclic AMP and dibutyryl cyclic AMP on the cell cycle

To observe the effects of exogenous cyclic AMP and dibutyryl cyclic AMP on the cell cycle, Meth-A cells were cultured in culture medium containing 25, 50 or 100 μM exogenous cyclic AMP or dibutyryl cyclic AMP. After 36 h of exposure to exogenous cyclic AMP or dibutyryl cyclic AMP, the cells were stained with propidium iodide and subjected to flow cytometry. Figure 2 shows the histograms. The first peak was produced by cells with a 2C, the second with a 4C and the third with an 8C DNA content.

Exogenous cyclic AMP did not affect the cell cycle even at the high dose of 100 μM (Figures 2b,c,d). However, the cell cycle was affected by dibutyryl cyclic AMP in a dose-dependent manner. Twenty-five micromolar dibutyryl cyclic AMP caused a slight increase of cells with 4C DNA content (Figure 2e). When the concentration of dibutyryl cyclic AMP was increased to 50 μM , the main peak of the control shifted from 2C to 4C (Figure 2f) and at 100 μM a marked peak of 8C appeared (Figure 2g).

Potential of K252a-induced polyploidization by low concentrations of exogenous cyclic AMP and dibutyryl cyclic AMP

Figure 3 shows the shift in DNA content following treatment with 1.00 μM K252a in the presence of low concentrations of exogenous cyclic AMP or dibutyryl cyclic AMP for 36 h. The potentiation of K252a-induced polyploidization by exogenous cyclic AMP was similar to that by dibutyryl cyclic AMP. At as little as 3.13 μM exogenous cyclic AMP or dibutyryl cyclic AMP, the main peak shifted from K252a-induced 4C to 8C and a small population of cells with a 16C DNA content was produced (Figures 3b,d). At a concentration of 12.50 μM , an increase in the 8C cells was evident (Figures 3c,e).

Effect of K252a on attachment

The potentiation of K252a-induced polyploidization by low concentrations of exogenous cyclic AMP suggested a functional change in the cell membrane after K252a treatment. To provide further evidence supporting this suggestion, the attachment of control and K252a-treated cells to the culture dishes was compared. Under normal conditions, Meth-A cells grow anchorage-independently in suspension, although a small number of cells are loosely attached to the culture dish. Using this test, Meth-A cells were cultured for 48 h and the number of attached cells was determined at various times. As shown in Figure 4, although the attachment of control cells was slowly increased, only 27.71% of the control cells were

Figure 2. Effects of exogenous cyclic AMP and dibutyryl cyclic AMP on the cell cycle. Meth-A cells were treated with 0 (a), 25 (b), 50 (c), or 100 μM (d) exogenous cyclic AMP, or 25 (e), 50 (f), or 100 μM (g) dibutyryl cyclic AMP for 36 h. The cells were collected and the distributions of DNA content were determined by flow cytometry.

attached at 48 h after culture. In contrast, as early as 6 h after treatment with 1.00 μM K252a, markedly increased cell attachment was detected and the number of attached cells reached 33.74%. Between 9 and 48 h, the attachment of K252a-treated cells increased gradually and reached 69.18% at 48 h.

Effect of K252a on morphology

In order to determine whether a structural change in the cell membrane is associated with K252a-induced functional changes, including the induction of responsiveness to exogenous cyclic AMP and the increase of attachment, the surfaces of cells treated with 1.00 μM of K252a for 36 h were visualized by scanning electron microscopy (Figure 5). As compared with control cells which were small, spherical and poorly spread, the following differences in K252a-treated cells were noted: the cell size was considerably enlarged; cell bodies became flattened and had a smoother surface appearance; and at the edges, small ruffles, microspike-like appendages and lamellipodia projecting from the cell body were observed.

DISCUSSION

In yeast, it has been reported that exogenous cyclic AMP can penetrate into cells and consequently affect cell cycle progression (Baroni *et al.* 1992), the activities of glucosamine-6-

Figure 3. Potentiation of K252a-induced polyploidization by exogenous cyclic AMP and dibutyryl cyclic AMP. Meth-A cells were treated with $1.00 \mu\text{M}$ K252a (a) in the presence of $3.13 \mu\text{M}$ (b), or $12.50 \mu\text{M}$ (c) exogenous cyclic AMP, or in the presence of $3.13 \mu\text{M}$ (d) or $12.50 \mu\text{M}$ (e) dibutyryl cyclic AMP for 36 h. The cells were collected and the distributions of DNA content were determined by flow cytometry.

Figure 4. Effect of K252a on attachment. Meth-A cells were treated with 0 or $1.00 \mu\text{M}$ K252a for 48 h and the numbers of unattached and attached cells were determined at various times using a Coulter Counter. The percentage of cells attached to the culture dishes is shown.

Figure 5. Effect of K252a on morphology. The surfaces of Meth-A cells treated with 0 (top) or 1.00 M (bottom) K252a for 36 h were visualized by scanning electron microscopy. Small ruffle is marked by arrowhead. A microspike-like appendage is marked by small arrow. Lamellipodia are marked by large arrows. Bar = 1.00 μm .

phosphate deaminase and *N*-acetylglucosamine permease, and protein synthesis (Singh *et al.* 1980). However, in eukaryotic cells, membrane-permeable cyclic AMP analogues are used to investigate the physiological effects of intracellular cyclic AMP in place of the relatively membrane-impermeable exogenous cyclic AMP (Busse *et al.* 1993). The present results were consistent with those of most previous experiments, showing that exogenous cyclic AMP did not interfere with the cell cycle even at the high dose of 100 μM , and that dibutyryl cyclic AMP positively controlled cell cycle progression and induced polyploidization at doses higher than 100 μM in Meth-A cells. However, the interesting result that as little as 3.13 μM exogenous cyclic AMP markedly potentiated K252a-induced polyploidization was uncovered,

with a dose-effect profile similar to that of dibutyryl cyclic AMP. Two possibilities, i.e. that the inhibition of K252a-sensitive protein kinase(s) by exogenous cyclic AMP directly enhances or indirectly enhances the induction of polyploidization, emerged. The latter is more likely to be responsible, because it was observed that exogenous cyclic AMP does not interfere with the cell cycle in the presence of low doses of K252a, which does not induce polyploidization (data not shown). In any case, the cell membrane appears to be of prime importance in the mechanism that allows exogenous cyclic AMP to exert pharmacological effects during the formation of polyploid cells.

Polyploidization is a complex process involving several distinct steps: escape from the mitotic block, a bypass of cellular division, entry into a new cycle of DNA replication and the characteristic induction of the formation of polyploid cells with $> 8C$ DNA content (Usui *et al.* 1991). It was hypothesized that there are changes in the cell membrane during the complex process of polyploidization induction. To explore this possibility, changes in membrane function and structure were investigated. Firstly, the attachment of cells after K252a treatment was examined as an indicator of cell membrane function: cells treated with K252a were found to adhere more rapidly and more efficiently to the culture dish. To look for structural changes, scanning electron microscopy was used to observe the cell surfaces, and it was found that K252a-treated cells and control cells had markedly different cell surfaces. These findings supported further the supposition that changes in the cell membrane resulted in the pharmacological effects of exogenous cyclic AMP.

Although the exact mechanisms whereby exogenous cyclic AMP exerts pharmacological effects during the formation of polyploid cells are not understood, there are several possibilities, including: (1) exogenous cyclic AMP penetrates into the cells, and accordingly elevates directly the level of the intracellular cyclic AMP; and (2) the site of action of exogenous cyclic AMP is the cell membrane, and exogenous cyclic AMP indirectly elevates the level of the intracellular cyclic AMP either through inhibition of the phosphodiesterase or stimulation of adenylate cyclase. Polyploidization induction is useful system to explore the exact mechanism. Further studies concerning the potentiation of K252a-induced polyploidization by exogenous cyclic AMP are now in progress in the authors' laboratory using this experimental system.

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