Possible role of calmodulin in stimulation of hexose transport by 12-O-tetradecanoylphorbol-13-acetate, a tumor promoter[1]

K. Yamanishi, H. Nishino and A. Iwashima

Department of Biochemistry, Kyoto Prefectural University of Medicine, Kyoto 602 (Japan), October 12, 1982

Summary. 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent tumor promoter stimulated the rate of 2-deoxy-D-glucose (2DG) transport. Three different kinds of calmodulin antagonists inhibited the TPA-stimulated 2DG transport, the mechanism of which was examined in kinetic analysis. These results indicate that the expression of the effect of TPA may depend on Ca^{2+}-calmodulin system.

A potent tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), interacts with plasma membranes to induce many biological events in vitro[2]. Stimulation of membrane transport of hexoses[3,4], amino acids[5,6] and ions[7] is among the earliest of the effects of TPA which have been suggested to play an essential role in the process of tumor promotion.

Some of the TPA-induced biological changes have been reported to depend on Ca^{2+}[8-10]. Many biological processes regulated by Ca^{2+} involve an interaction with calmodulin[11], and it has also been shown that Ca^{2+}-calmodulin complex mediates several of the effects of TPA on plasma membrane[12-14].

In this communication, we provide evidence suggesting that TPA stimulates hexose transport in chick embryo fibroblasts via Ca^{2+}-calmodulin system.

Materials and methods. Cell culture. Chick embryo fibroblasts were prepared from 10-day-old embryos as described previously[15], and grown in monolayers in Eagle's minimum essential medium supplemented with 10% newborn calf serum at 37 °C in a humidified CO_2 incubator and plated at 7×10^5 cells/35 mm diameter dishes. After 2 days, cultures attaining confluence were used in the study.

Assay of 2DG uptake. Chick embryo fibroblasts were treated appropriately, incubated at 37 °C for a designated period of time, and then rinsed once with 2 ml of phosphate buffered saline (PBS). The uptake was initiated by the addition of 1 ml of PBS containing 4 μM of 2-deoxy-[^{3}H]-glucose (2 μCi/ml) at room temperature. After a definite time 2DG uptake was stopped by washing the plates 3 times with 2 ml of PBS. The cells were dissolved in 1 ml of 0.1 M NaOH containing 0.1% sodium dodecyl sulfate, and portions of the lysate were taken for assay of radioactivity and for protein determination[16]. Duplicate or triplicate plates were employed for each experiment, which was performed at least 3 times. The linearity of 2DG uptake was maintained for 20 min, and TPA (50 nM) induced a 2-fold increase in 2DG transport within 4 h, as reported previously[3,4].

Statistical estimation. The decrease in TPA-stimulated 2DG uptake induced by each calmodulin antagonist was expressed as the mean ± SEM (3 separate experiments). Kinetic parameters K_m and V_{max} were determined using weighted fits of Lineweaver-Burk plots, with the individual mean rates of triplicate experiments weighted by the factor $V^4/(SE)^2$ and the SE for K_m and V_{max} were calculated as described by Wilkinson[17]. The values of these parameters were expressed as the mean ± SEM. Significant differences were assessed using the 2-tailed Student t-test.

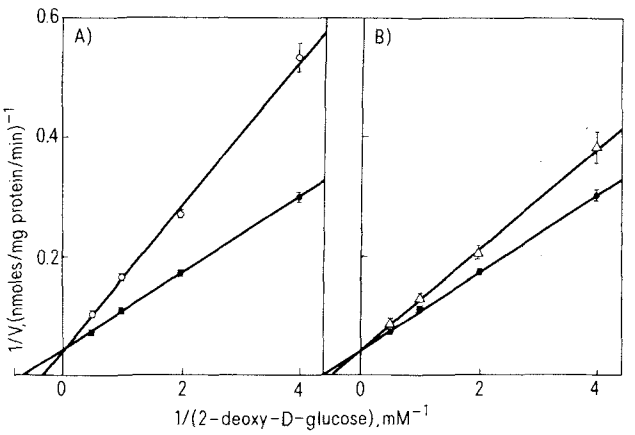
Materials. 2-deoxy-[^{3}H]-glucose (5 Ci/mmole) was purchased from New England Nuclear and newborn calf serum was from GIBCO. Trifluoperazine · HCl was a gift from Yoshitomi Pharmaceutical Co., Ltd and prenylamine · lactate was from Daika Co., Ltd. N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and N-(6-aminohexyl)-1-naphthalenesulfonamide (W-5) were obtained from Rikaken Co. 12-O-tetradecanoylphorbol-13-acetate (TPA) was purchased from P-L Biochemicals Inc.

Results and discussion. The involvement of calmodulin in the effect of TPA on hexose transport was probed with various calmodulin antagonists. Chick embryo fibroblasts were preincubated for 1 h with each calmodulin antagonist, followed by addition of 50 nM TPA, and 4 h later 2DG uptake for 5 min was assayed as described in 'Materials and methods'.

Trifluoperazine, a phenothiazine derivative[18], inhibited TPA-stimulated 2DG uptake in a dose-dependent manner. The concentration of trifluoperazine required to inhibit the TPA-stimulated 2DG uptake by 50% (IC_{50}) was 25 μM (decrease in 2DG uptake: 9.4±2.2 pmoles/mg protein/min; $n=3$; $p<0.05$). At concentrations above 50 μM, trifluoperazine showed a severe cytotoxic effect - cells were detached from plates.

Calmodulin antagonists, however, can affect processes other than those which are Ca^{2+}-calmodulin dependent. Induction of ornithine decarboxylase activity by TPA, for example, was inhibited by trifluoperazine[19], but not by another calmodulin antagonist, indicating that the Ca^{2+}-calmodulin system is not necessarily involved in the ornithine decarboxylase induction by TPA[20]. Therefore, different calmodulin antagonists were used in order to investigate the regulation of a biological process by calmodulin.

Another calmodulin antagonist W-7, an N-naphthalenesulfonamide derivative, inhibited the effect of TPA in concentrations up to 100 μM in dose-dependent fashion without affecting the basal uptake of 2DG, and IC_{50} of W-7 was about 50 μM (decrease in 2DG uptake: 8.6±0.87 pmoles/mg protein/min; $n=3$; $p<0.01$). On the other hand, W-5,



A Lineweaver-Burk plots of the initial rates of 2DG uptake in the presence or absence of TPA. Chick embryo fibroblasts were incubated for 4 h with 50 nM TPA (●) or with 0.2% dimethylsulfoxide (○) as solvent control, and then hexose transport was assayed. *B* Lineweaver-Burk plots of the initial rates of TPA-stimulated 2DG uptake in the presence or absence of W-7. Chick embryo fibroblasts were preincubated for 1 h in the presence (△) or absence (●) of 75 μM W-7, and then 50 nM TPA was applied. 4 h later, hexose transport was assayed. For kinetic analysis, 2DG uptake for 2 min at concentrations between 0.25 and 2 mM was determined. Data represent the mean ± SEM of triplicate experiments.

which is a chlorine-deficient analogue of W-7 which only weakly interacts with calmodulin[21], did not inhibit the TPA-stimulated 2DG uptake at concentrations up to 100 μM. Prenylamine, a N-diphenylpropyl derivative of amphetamine, which was reported to antagonize calmodulin[22], also inhibited 2DG uptake stimulated by TPA. IC_{50} of prenylamine was about 25 μM (decrease in 2DG uptake: 10 ± 2.2 pmoles/mg protein/min; $n = 3$; $p < 0.05$). These inhibitory effects of calmodulin antagonists on TPA-stimulated 2DG uptake correlate well with the IC_{50}-values for inhibition of Ca^{2+}-dependent phosphodiesterase activity reported previously[18,21].

To analyze the effect of TPA and calmodulin antagonists on hexose transport, kinetic studies were carried out. Lineweaver-Burk plots of the initial rates of 2DG uptake in the presence or absence of TPA are shown in the figure, A. Chick embryo fibroblasts treated with TPA had significantly decreased K_m for 2DG transport compared to controls (1.43 ± 0.02 vs 2.77 ± 0.25 mM; $n = 4$; $p < 0.01$), but the V_{max}-values were not significantly different (22.7 ± 0.24 vs 23.5 ± 1.4 nmoles/mg protein/min). Thus, in chick embryo fibroblasts, TPA stimulates hexose transport by changing the affinity for the substrate, but not by affecting the number of hexose functional carriers. This is different from the properties in virus-transformed chick embryo fibroblasts in which V_{max} is increaed without change of K_m[23].

For analysis of the kinetics of the inhibitory action of calmodulin antagonists on TPA-stimulated 2DG uptake, W-7 was selected as a reliable antagonist because among calmodulin antagonists only W-7 was proved to penetrate through cell membrane and be distributed in cytoplasm[21]. The figure B shows Lineweaver-Burk plots of the initial rates of TPA-stimulated hexose transport in the presence or absence of W-7. In TPA-treated fibroblasts, W-7 significantly increased K_m compared to control (1.91 ± 0.13 vs 1.43 ± 0.02 mM; $n = 4$; $p < 0.01$) without affecting V_{max} (23.4 ± 1.0 vs 22.7 ± 0.24 nmoles/mg protein/min). In other words, a calmodulin antagonist W-7 reversed the decreased K_m induced by TPA. Therefore, W-7 may specifically affect the mechanism through which TPA induces the stimulation of hexose transport.

From the results obtained in this study, we suggest that the effect of TPA on hexose transport in chick embryo fibroblasts may be mediated by Ca^{2+}-calmodulin system.

1 Acknowledgment. This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

2 Diamond, L., O'Brien, T.G., and Baird, W.M., Adv. Cancer Res. *32* (1980) 38.
3 Driedger, P.E., and Blumberg, P.M., Cancer Res. *37* (1977) 3257.
4 Lee, L.S., and Weinstein, I.B., J. Cell Physiol. *99* (1979) 451.
5 Nishino, H., Nishino, A., Fujita, K., Hasegawa, T., and Iwashima, A., Cell Struct. Funct. *5* (1980) 265.
6 Becker, J.H., and Cook, J.S., Cancer Res. *41* (1981) 4512.
7 Dicker, P., and Rozengurt, E., Biochem. biophys. Res. Commun. *100* (1981) 433.
8 Wenner, C.E., Hackney, J., Kimelberg, H.K., and Mayhew, E., Cancer Res. *34* (1974) 1731.
9 Fisher, P.B., and Weinstein, I.B., Carcinogenesis *2* (1981) 89.
10 Nishino, H., Fujita, K., Inoue, F., Hasegawa, T., and Iwashima, A., J. Kyoto Pref. Univ. Med. *90* (1981) 547.
11 Cheung, W.Y., Science *207* (1980) 19.
12 Murray, A.W., Jones, M.J., Guy, G.R., and Froscio, M., Biochem. int. *3* (1981) 1.
13 Froscio, M., Guy, G.R., and Murray, A.W., Biochem. biophys. Res. Commun. *98* (1981) 829.
14 Kwong, C.H., and Mueller, G.C., Cancer Res. *42* (1982) 2115.
15 Adams, R.L.P., in: Cell culture for biochemists, p.79. Ed. R.L.P. Adams. Elsevier/North-Holland Biomedical Press, Amsterdam 1980.
16 Markwell, M.A.K., Hass, S.M., Bieber, L.L., and Tolbert, N.E., Analyt. Biochem. *87* (1978) 206.
17 Wilkinson, G.N., Biochem. J. *80* (1961) 324.
18 Hidaka, H., and Tanaka, T., in: Calmodulin and intracellular Ca^{2+} receptors, p.19. Eds S. Kakiuchi, H. Hidaka and A.R. Means. Plenum Press, New York 1982.
19 Verma, A.K., and Boutwell, R.K., Biochem. biophys. Res. Commun. *101* (1981) 375.
20 Naito, E., Kodama, T., Takino, T., and Nishino, H., J. Kyoto Pref. Univ. Med. *92* (1983) 83.
21 Hidaka, H., Sasaki, Y., Tanaka, T., Endo, T., Ohno, S., Fujii, Y., and Nagata, T., Proc. natl Acad. Sci. USA *78* (1981) 4354.
22 Honda, F., Katsuki, S., and Sakai, N., Jap. J. Pharmac., suppl. *23* (1973) 27.
23 Inui, K., Tillotson, L.G., and Isselbacher, K.J., Biochim. biophys. Acta *598* (1980) 616.

0014-4754/83/091036-02$1.50 + 0.20/0

Effect of X-ray irradiation on developing eggs of the silkworm

S. Mathavan

School of Biological Sciences, Madurai Kamaraj University, Madurai-625 021 (India), March 3, 1982

Summary. Eggs of *B. mori* were exposed to X-ray irradiation at different times after laying, and the changes in fertilization, hatchability and mortality of the eggs were observed. Percentage of fertilization and hatchability increased with increasing age of the egg. Statistical analyses of the data show that the observed changes are age-dependent.

Insects, particularly silkworms, have been considered to be good tools for observation of genetic and nongenetic effects of ionizing radiations[1,2]. Several workers have exposed the eggs and larvae of the silkworm to varying doses of X-ray, γ-ray and UV-irradiations to obtain a dominant mutant as well as to demonstrate the radio-sensitivity of different stages of the silkworm[3-6]. Eggs of the silkworm *Bombyx mori* (Indian race) were exposed to different doses of X-ray irradiation for mutation studies and the present paper reports the observations made on the effect of X-ray irradiation on the eggs of the silkworm, at various stages of development.

Materials and methods. The silkworm chosen for the present study was an Indian race, Pure Mysore. Layings were prepared on egg cards from the freshly emerged healthy moths. Disease free layings of 1-, 3-, 6-, 9-, 12- and 24-h-old eggs were chosen for irradiation studies. The eggs laid by the moth during the first hour from the initiation of egg laying were described as 1-h-old, and the age of the eggs was determined similarly in other cases. In fact, 1- and 3-h-