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EFFECTS OF DIBUTYRYL ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE ON HUMAN MELANOCYTES IN VITRO

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Abstract. The effects of cyclic AMP and its analogue, dibutyryl cyclic AMP (DBcAMP) on the pigmentary system were studied by using human epidermal melanocytes in culture. The melanocytes responded to 1 mM DBcAMP with an increase in number, length, and complexity of dendritic processes. The effect of DBcAMP on the dendritogenesis was reversible. Melanin synthesis, as indicated by the uptake of tyrosine in the presence of an inhibitor of protein synthesis, was significantly stimulated by DBcAMP. The maximum stimulation was observed at concentrations of 0.5 mM, and 1.0 mM. The melanin synthesis increased after 12-hour treatment with DBcAMP and continued to increase with the prolonged treatment. Cyclic AMP, theophylline, sodium butyrate, or 5′-AMP at a concentration of 1 mM did not have any remarkable effect on the morphology or the melanin synthesis of the melanocyte. The results of this investigation indicate the possible role of the MSH-cyclic AMP system in the melanin pigmentation of human skin and represent a system for further study of the pathobiology of human melanocytes.

Key words: Dibutyryl cyclic AMP; Melanocyte; Cell Culture

Adenosine 3′,5′-cyclic monophosphate (cyclic AMP) appears to be the "second messenger" for a variety of hormonal effects. Evidence that the effects of epinephrine, glucagon, ACTH, TSH, vasopressin, and parathyroid hormone are mediated by cyclic AMP is now substantial.

Sutherland and his colleagues have established four criteria that should be satisfied in order to justify a claim that a hormone produces its effect via an alteration in the intracellular level of cyclic AMP (19). Evidence that the effect of melanocyte-stimulating hormone (MSH) is mediated by cyclic AMP is summarized as follows. First, MSH caused an increase in cyclic AMP (1). Second, the inhibitor of phosphodiesterase acted synergetically with MSH (1). Third, the effect of MSH can be mimicked by exogenous cyclic AMP or its dibutyryl derivative (1, 2, 3, 6, 14, 15, 20). Therefore it is more than likely that the effect of MSH is mediated by cyclic AMP. However, the stimulation of adenyl cyclase activity by MSH in broken cell preparations remains to be proved.

These evidences were demonstrated mostly in amphibian melanophores by using the rapid dispersion of melanin granules as an indicator. It is now necessary to investigate the effect of cyclic AMP on the mammalian melanocyte. The purpose of this communication is to present data which show that the cyclic AMP analogue, dibutyryl cyclic AMP, stimulates formation of dendritic processes and melanin synthesis of human epidermal melanocytes.

MATERIALS AND METHODS

Cell cultures of adult human epidermis were established according to a method described previously (9). Briefly, dermatome-split human skin was treated with 0.02% EDTA solution for 5 min at room temperature and kept in 0.25% trypsin solution for 12 to 24 hours in a refrigerator (4°C). Thereafter, the epidermis was separated and the epidermal cells were mechanically dispersed with fine forceps. After centrifugation and resuspension in a culture medium, the cells were distributed into 60×15 mm plastic culture dishes (Falcon Plastic). These were incubated in a moisture-controlled chamber with 5% CO_2 in air at 37°C. The medium consisted of Eagle's minimal essential medium supplemented with 30% calf serum. For autoradiography, several drops of the epidermal cell suspension were placed on a coverglass in the culture dish and were incubated in the chamber. After 24 hours of incubation, when the cells were firmly attached to the coverglass, 4 ml of the medium was added to the dish.

Sufficient N^6-2′-*O*-dibutyryl adenosine 3′,5′-monophosphoric acid monosodium salt (DBcAMP) (Daiichi Chemicals Co.), adenosine 3′,5′-cyclic monophosphate (cAMP) (Sigma Chemicals Co.), sodium *n*-butyrate (NaB) (Nakarai Chem.), adenosine 5′-monophosphate (5′-AMP) (Kohjin Biochem.), or theophylline (Th) (Sigma Chemicals Co.) was dissolved in distilled water so that the addition of 0.1 or 0.2 ml of the solutions to a dish containing 4 ml of the medium gave the final concentration used in the experi-

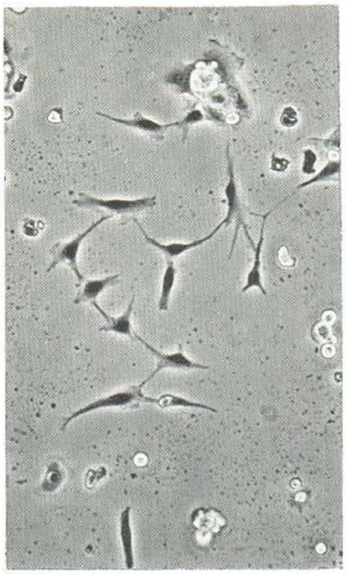

Fig. 1. Keratinocytes and melanocytes in culture. At the top of the figure, a few keratinocytes touch each other. Melanocytes look darker and have processes. Untreated control. 3-day culture. (Phase contrast, ×150.)

ments. The chemicals were added to the culture medium following a few days of cultivation. When prolonged treatments were required, the medium was changed every 2 days and fresh reagents were added. Serial pictures of the selected areas were photographed with a camera attachment to a Nikon inverted phase contrast microscope.

For autoradiography, 5 μCi/ml ^{3}H-tyrosine (L-tyrosine-3,5-^{3}H, New England Nuclear Corp., specific activity 70 Ci/mM) was added to the culture medium and the cultures were incubated for 2 hours. To inhibit protein synthesis, 250 μg/ml cycloheximide (Nakarai Chem.) was added 30 min before the addition of the radioisotope. After the radioisotope treatment, the cultures were washed with Ringer solution, fixed with methyl alcohol and air-dried. The specimens were dipped in Sakura NR-M2 liquid emulsion, exposed for 2 weeks, developed and stained with Mayer hematoxylin. In the pigment cells tyrosine was specifically incorporated into melanin in the presence of a potent inhibitor of protein synthesis (10). Melanin synthesis was determined by counting grains over 100 melanocytes. For statistical analysis, the number of the grains was converted into logarithms in order to correct the pattern of distribution. A test of significance was done by analysis of variance and multiple comparison (Tukey's test and Scheffe's test).

RESULTS

Morphological observation

In 2 or 3 days after the initiation of the culture the keratinocytes coalesced to form a sheet. The melanocytes scattered singly or in groups around the sheet of keratinocytes (Fig. 1). DBcAMP at a concentration of 1 mM caused a marked increase in number, length, and complexity of dendritic processes of the melanocyte (Fig. 2). Within several hours after the addition of DBcAMP the process began to elongate and increased in number. The length of the process often exceeded that of the cell body and secondary and tertiary branchings appeared. In the control culture the length of the process usually did not exceed the length of the cell body and secondary and tertiary branchings were observed only rarely (Fig. 1). After one week of treatment with 1 mM DBcAMP the melanocytes took on a polygonal shape, became larger and looked darker (Fig. 3). The effect of DBcAMP on the dendritogenesis was reversible. Within a few hours after the removal of the culture medium containing DBcAMP, and the renewal of the fresh medium lacking DBcAMP, the elongated dendritic process became shorter (Figs. 4, 5). Cyclic AMP, Th, NaB, or 5′-AMP at a concentration of 1 mM had no remarkable effects on the morphology of the melanocyte.

Autoradiographic study

There was marked increase in melanin synthesis of DBcAMP-treated melanocytes (Fig. 6). In the control culture the average number of grains per

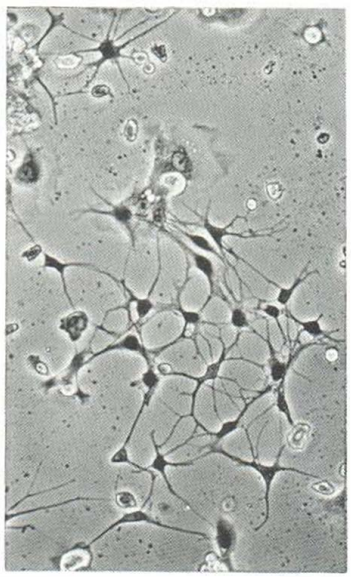

Fig. 2. DBcAMP treatment for 48 hours. Numerous dendritic processes with secondary and tertiary branchings. (Phase contrast, ×150.)

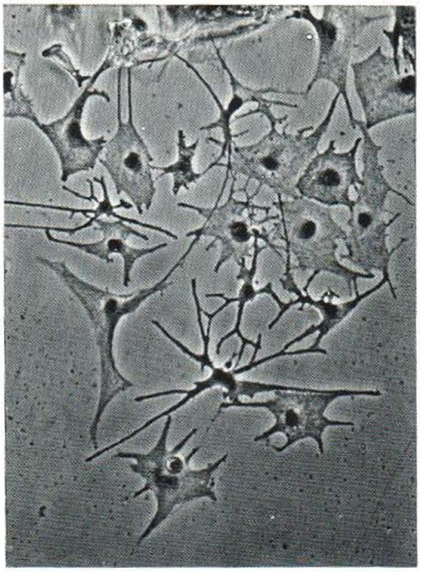

Fig. 3. DBcAMP treatment for 7 days. The melanocytes become larger and take on a polygonal shape. (Phase contrast, ×150.)

melanocyte was 9.2 and only 14% of the melanocytes had more than 15 grains. Whereas in the culture treated with 1 mM DBcAMP for 48 hours the average numbers of grains counted was 59.1, 95% of the melanocytes had more than 15 grains and the distribution of number of grains per melanocyte was wide ranging, from 2 to 241. In the culture treated with 1 mM cAMP, or 1 mM Th for 48 hours the average numbers of grains was 12.6 and 13.2 respectively. The difference between the control and the DBcAMP-treated cultures was highly significant statistically. The differences between the control,

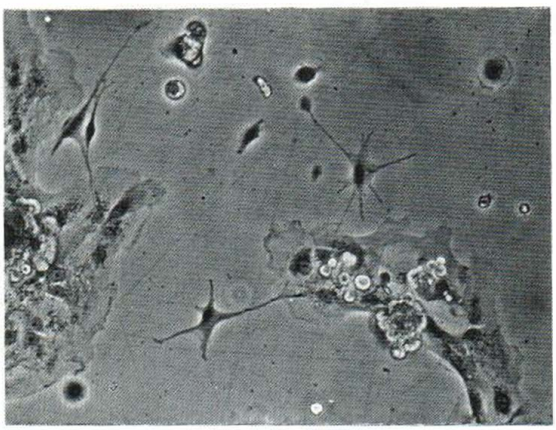

Fig. 4. DBcAMP treatment for 24 hours. Before the removal of DBcAMP. (Phase contrast, ×150.)

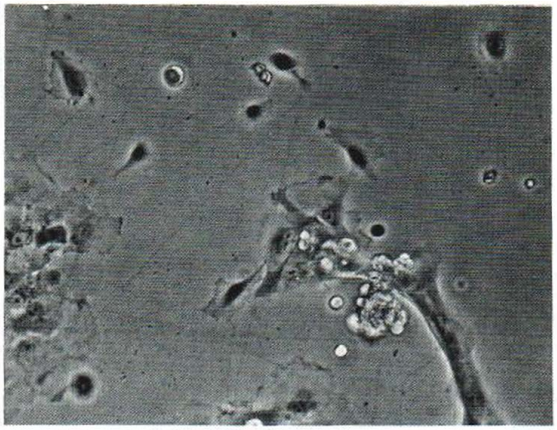

Fig. 5. The same field as Fig. 4. 8 hours after the removal of the medium containing DBcAMP, and the renewal of the fresh medium lacking DBcAMP. Note disappearance of dendritic processes of melanocytes. (Phase contrast, ×150.)

cAMP-treated, and Th-treated cultures were not significant.

Fig. 7 indicates that melanin synthesis was enhanced after 24 hours of treatment with 0.2, 0.5, 1.0, and 2.0 mM DBcAMP. The maximum enhancement was observed at 0.5, and 1.0 mM. The numbers of grains per melanocyte averaged 24.7, and 24.7 (control: 12.5) respectively. The enhancement of melanin synthesis appeared to be dose-dependent between 0.1, and 0.5 mM. At 2.0 mM melanin synthesis

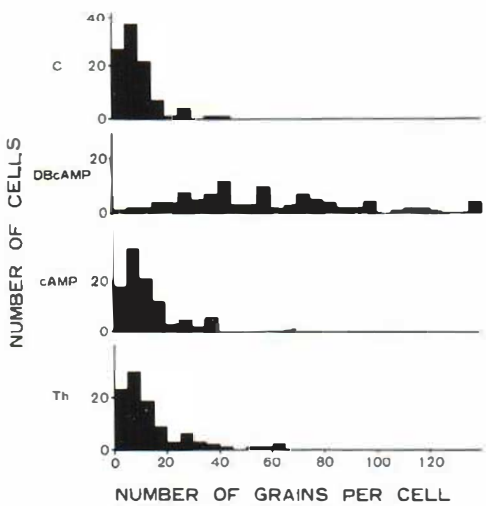

Fig. 6. Effect of DBcAMP, cAMP, and Th on melanin synthesis. The cultures were treated with the chemicals at a concentration of 1 mM for 48 hours and were exposed to ^{3}H-tyrosine in the presence of cycloheximide. *C:* control.

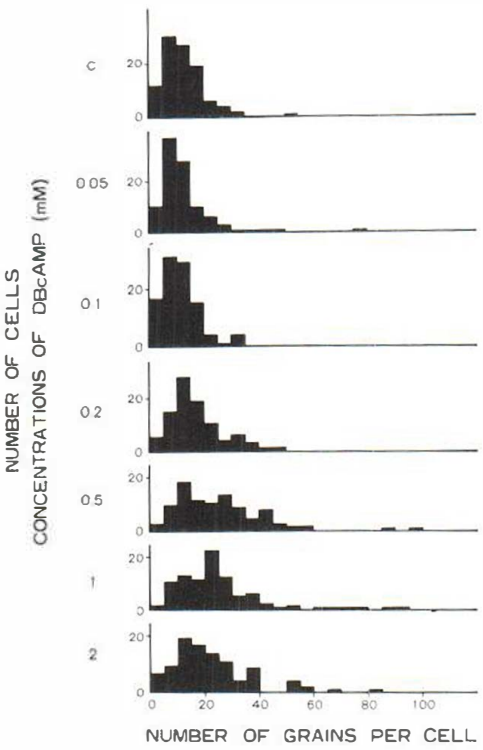

Fig. 7. Effect of various concentrations of DBcAMP on melanin synthesis. The cultures were treated with DBcAMP at various concentrations for 24 hours and were exposed to ^{3}H-tyrosine in the presence of cycloheximide. *C:* control.

was slightly reduced (21.5). Melanin synthesis increased with prolonged treatment up to 48 hours (Fig. 8). When 1 mM DBcAMP was added to the culture simultaneous with ^{3}H-tyrosine (0 h), melanin synthesis did not increase (average numbers of grains, Control: 4.3; 0 h: 3.7). After 12 hours of the treatment, melanin synthesis increased (7.5) and this increase was statistically significant. The melanin synthesizing activity further increased 24, and 48 hours (14.8, and 17.7) later.

To test the specificity of the DBcAMP-induced activation of melanin synthesis for the cyclic adenosine nucleotide portion of the molecule, the effects of butyrate (a product of the deacetylation reaction), and 5'-AMP (a noncyclic adenosine nucleotide) were bioassayed. Figs. 9 and 10 indicate that neither NaB nor 5'-AMP stimulated melanin synthesis.

DISCUSSION

In 1965 Bitensky & Burstein reported that cAMP caused darkening of excised strips of frog skin, as did MSH (2). Novales & Davis made a similar study to show darkening of frog skin and melanin dispersion within tissue cultured melanophores (14). Almost contemporaneously, many workers reported the darkening effect of exogenous cAMP on amphibian and fish skin (1, 6, 15, 20). These results were discussed in terms of the possibility that cAMP may be the intracellular mediator of MSH actions. In 1969 Abe et al. reported that MSH stimulated cAMP formation in the dorsal skin of *Rana pipiens* (1). Graded concentrations of α-MSH produced graded increases in cAMP levels. The darkening potencies of α-MSH, β-MSH, and ACTH were correlated to their capacity to stimulate cAMP accumulation. The increased cAMP levels occurred at least as fast as darkening in response to MSH. Van de Veerdonk & Konijn found a higher level of cAMP in frog skin adapted to black background (20). Chen & Tchen reported that the melanocytogenic activity of MSH could be duplicated by DBcAMP in organ cultures of

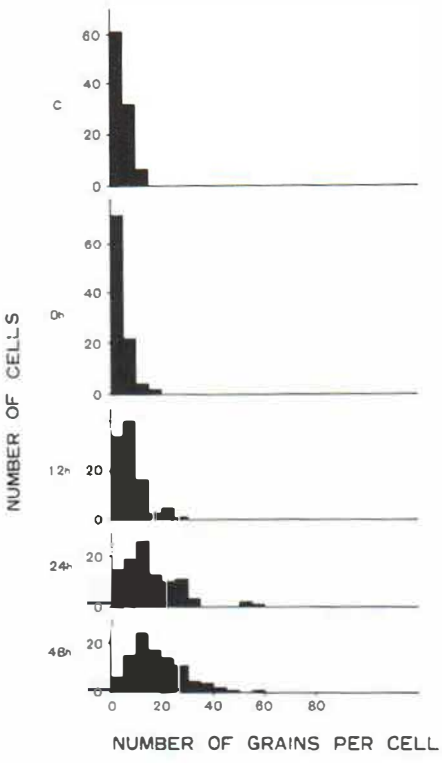

Fig. 8. Time course of stimulation of melanin synthesis by DBcAMP. The cultures were treated with 1 mM DBcAMP for 1, 12, 24, and 48 hours and were exposed to ^{3}H-tyrosine in the presence of cycloheximide. *C:* control.

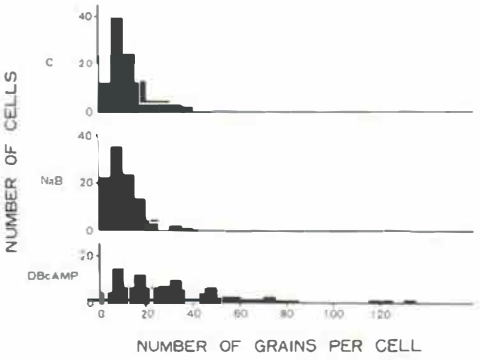

Fig. 9. Effect of NaB, and DBcAMP on melanin synthesis. The cultures were treated with NaB, and DBcAMP at a concentration of 1 mM for 48 hours and were exposed to ^{3}H-tyrosine in the presence of cycloheximide. *C:* control.

xantheic goldfish tailfins (3). Substantial evidence that the effects of MSH on melanophores were mediated by cAMP was thereby accumulated.

The present study showed that DBcAMP at a concentration of 1 mM stimulated elongation and branching of the dendritic processes of human epidermal melanocytes. Novales & Davis observed expansion of amphibian melanophores 10 min after the addition of 10 mM cAMP. The elongation of dendritic processes of the human melanocyte—an analogous phenomenon to the expansion of the amphibian melanophore—occurred at slower rate. Snell demonstrated that MSH caused an increase in the length, width, and complexity of the dendritic processes of guinea pig epidermal melanocytes (17, 18). The in vitro study on the reaction of mammalian epidermal melanocyte to MSH was fruitless. Hu introduced MSH into cultures of melanoma cells without dispersing melanin granules (7). Klaus & Snell reported that no change in either the cell morphology or melanin granule activity was observed in guinea pig epidermal melanocytes in culture after the addition of MSH (11). For the first time in the literature the author showed elongation and branching of dendritic processes of the mammalian epidermal melanocyte in culture in response to a chemical or a hormone.

There are relatively few reports on the participation of MSH in melanin synthesis. Dawes proved by a colorimetric method that the melanin content of frog skin was increased or decreased when frogs were housed against a black or a white background (4). He also showed an increase in the skin's melanin content following intramuscular injection of pituitary extract. Frieden & Bozer reported that the administration of pituitary extract to frogs resulted in an initial decrease in melanin content, and this was then followed by a slow increase (5). After 8 weeks of daily injection of the extract, melanin content reached 40% above that of the control animals. In guinea pigs, the amount of free melanin and of melanin within melanocytes was found to increase after 4 weeks of injection of MSH (17, 18). In humans, the melanocytes showed a marked increase in melanin content after 1 week of treatment with MSH and an increase in the amount of free melanin was noted after 23 weeks (13). Recently Wong & Pawelek reported that MSH caused a substantial increase in tyrosinase activity and in melanin content of a mouse melanoma cell line (Cloudman S91, NCTC 3960) in monolayer culture and that cAMP substituted for MSH (21). Johnson & Pastan obtained similar results with DBcAMP in the same melanoma cell line (8). The results of the study conducted by Kreider et al. indicated that cAMP, DBcAMP, caffeine, and theophylline inhibited the growth of mouse melanoma cells (B16) and stimulated melanogenesis (12).

In the present study the melanocytes responded to DBcAMP with increased melanin synthesis, as was

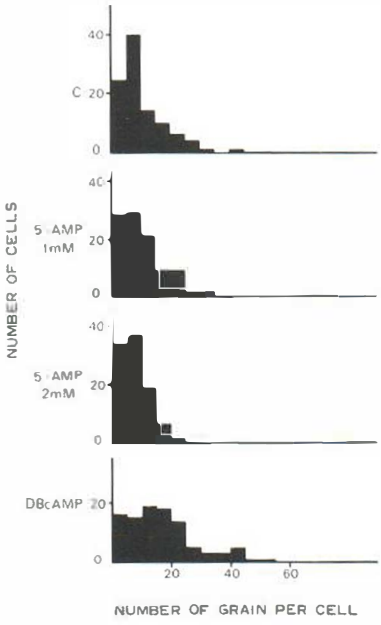

Fig. 10. Effect of 5'-AMP, and DBcAMP on melanin synthesis. The cultures were treated with 1 mM and 2 mM 5'-AMP, and 1 mM DBcAMP for 48 hours and were exposed to ^{3}H-tyrosine in the presence of cycloheximide. *C:* control.

demonstrated by the incorporation of tyrosine in the presence of cycloheximide, a potent inhibitor of protein synthesis. The increase in melanin synthesis appeared to be dose-dependent between the concentrations 0.1 and 0.5 mM and the maximum increase was reached at 0.5 mM, and 1.0 mM. The addition of DBcAMP to the culture did not stimulate melanin synthesis immediately. The melanin synthesis increased after 12 hours of treatment and continued to increase with prolonged treatment up to 48 hours. This indicates that DBcAMP did not stimulate tyrosinase directly but affected the system of melanin synthesis in a series of complicated steps.

Cyclic AMP at a concentration of 1 mM did not stimulate melanin synthesis. This might be explained by the weak entry of cAMP into cells and inactivation of cAMP by phosphodiesterase (19). DBcAMP is more lipid soluble than cAMP and hence is thought to penetrate the intact cell membrane better than cAMP (16, 19). DBcAMP is more resistant to inactivation by purified phosphodiesterase than cAMP (16). NaB, and 5'-AMP did not increase melanin synthesis. Thus the specificity of the DBcAMP-induced stimulation of melanin synthesis was corroborated.

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