Action of Cyclic AMP on Pigment Donation Between Mammalian Melanocytes and Keratinocytes

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Maintenance of normal skin coloration in mammals involves the manufacture of melanosomes within melanocytes and their transfer to surrounding keratinocytes. In vitro, it appears that portions of a melanocyte dendrite are pinched off and transferred to an associated keratinocyte (1-3). The mechanisms involved in this process are unclear. A band of microfilaments just beneath the cell membranes of both keratinocytes and melanocytes has been implicated as being involved in this transfer process (3).

Adenosine 3',5'-cyclic monophosphate (cAMP) is an important intracellular regulatory agent which acts to control the rate of a number of cellular processes (4). These include endocytosis of colloid by thyroid follicular cells (5-7), pinocytosis in the toad bladder (8), release of insulin from pancreatic islet cells (9-11), and melanosome movements in melanophores (12-16). Intracellular levels of cAMP are thought to be regulated by external stimulation of sites or receptors on the cell membrane. Stimulation of various receptors in turn leads to changes in adenyl cyclase activity and cAMP levels (4).

The present work was undertaken to observe the effects of dibutyryl cyclic AMP (DBcAMP) and the cAMP phosphodiesterase inhibitor, theophylline, on the rate of pigment donation from mammalian melanocytes to keratinocytes, studies were also undertaken to see if various agents known to stimulate α- or β-adrenergic receptors had any effect on this rate of transfer.

MATERIALS AND METHODS

Cell cultures of adult guinea pig ear epidermis were established in Cruickshank chambers and plastic Cooper dishes as previously described (3). Cells were grown in Eagle's minimal essential media containing 10% calf serum supplemented with 20% fetal calf serum and were incubated at 37°C. Cultures 7 to 14 days old were treated with the various test solutions each of which was made up in the culture

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media. Control cultures were treated with just a change of the normal media. Test substances included dibutyryl adenosine 3',5'-monophosphate, isoproterenol, propranolol, and epinephrine, all from Sigma Chemical Co., St. Louis, MO. Theophylline came from Nutritional Biochemical Corp. Cleveland, OH.

The cells were observed by phase-contrast microscopy, and their behavior recorded by time lapse cinemicrography. The rate of pigment donation was measured as the number of melanosome packets transferred per unit time. After light microscopic observation, the cultures were fixed for electron microscopy in 3% glutaraldehyde followed by 1% osmium tetroxide. Cells were then treated en bloc with 1% uranyl acetate in maleate buffer, dehydrated in a graded series of alcohols, and embedded in Epon (3). Thin sections were cut parallel to the surface of the dish, stained with uranyl acetate and lead citrate, and examined with an RCA-3G electron microscope.

RESULTS

Dibutyryl cyclic AMP, at both 8 mM and 10 mM concentrations, significantly increases the rate of pigment donation as compared to the untreated cells, $p < 0.01$ (Figs. 1 and 2). Dibutyryl cyclic AMP also increases the percent of melanocytes donating pigment (Table 1). The cAMP phosphodiesterase inhibitor, theophylline, likewise produces an increase in the rate of pigment donation (Fig. 2). This sub-maximal dose of theophylline (5 mM) in combination with 8 mM DBcAMP enhances the effect of DBcAMP on the rate of pigment donation (Fig. 2). Theophylline and theophylline plus DBcAMP also increase the percent of melanocytes donating pigment as compared to the controls (Table 1).

The $\beta$-adrenergic stimulator, isoproterenol, produces an increase in the rate of melanosome transfer. Figure 3 shows that 5 mM isoproterenol significantly increases the rate of transfer as compared to the control, $p < 0.01$. Isoproterenol also produces an increase in the percentage of melanocytes donating pigment (Table 2). The $\beta$-adrenergic antagonist propranolol blocks the effects of iso-

![Fig. 1. Effect of 10 mM DBcAMP on the rate of pigment donation from melanocytes to keratinocytes. Treatment in DBcAMP (●), 43 cells; media without DBcAMP (○), 43 cells. Points are means ± SE.](image-url)
Fig. 2. Effect of DBcAMP and theophylline on the rate of pigment donation from melanocytes to keratinocytes. Treatment in 8 mM DBcAMP (●), 5 mM theophylline (▲), 8 mM DBcAMP plus 5 mM theophylline (●), media only (○). Points are means ± SE for 47 cells.

TABLE 1
EFFECT OF DIBUTYRYL CYCLIC AMP AND THEOPHYLLINE ON THE PERCENT OF MELANOCYTES DONATING PIGMENT

| Agent                        | % of melanocytes donating pigment |
|------------------------------|----------------------------------|
|                              | No. of cells observed | Length of treatment |
|                              |                      | 2 hr | 3 hr |
| DBcAMP (10 mM)              | 43                   | 74   | 84   |
| Control                      | 43                   | 37   | 44   |
| DBcAMP (8 mM)               | 47                   | 73   |      |
| Theophylline (5 mM)         | 47                   | 60   |      |
| DBcAMP (8 mM) plus theophylline (5 mM) | 47 | 69   |      |
| Control                      | 47                   | 42   |      |

proterenol. The rate of pigment donation in cultures pretreated for 30 min with 0.1 mM propranolol, followed by treatment in 5 mM isoproterenol plus 0.1 mM propranolol, is similar to that of the controls (Fig. 3). Propranolol also inhibits the increase in the percent of melanocytes donating pigment which is produced by isoproterenol (Table 2).

The α-adrenergic stimulator epinephrine, on the other hand, had no effect on the rate of melanosome transfer. In cells treated with 3 mM and 5 mM epinephrine, the rate of donation was similar to that of the control (Fig. 4). Epinephrine, likewise, had no stimulatory effect on the percent of melanocytes donating pigment (Table 2).

Dibutyryl cyclic AMP, theophylline, and isoproterenol not only produce an increase in the rate of melanosome transfer, but they also produce an increase in the ruffling of the keratinocyte cell membrane. This activity is particularly noticeable in the area of contact with a melanocyte. Figure 5 was taken from a time-lapse film of an untreated culture. Figure 5a shows a melanocyte and keratinocyte. Fig-
Fig. 3. Effect of isoproterenol and propranolol on the rate of melanosome transfer from melanocytes to keratinocytes. Treatment in 5 mM isoproterenol (●), 0.1 mM propranolol for 30 min followed by 5 mM isoproterenol plus 0.1 mM propranolol (■), media only (○). Points are means ± SE for 34 cells.

### TABLE 2

**Effect of Isoproterenol and Epinephrine on Pigment Donation Between Mammalian Melanocytes and Keratinocytes**

| Agent                        | No. of cells observed | % of melanocytes donating pigment<sup>a</sup> |
|------------------------------|-----------------------|----------------------------------------------|
| Isoproterenol (5 mM)         | 34                    | 76                                           |
| Propranolol (0.1 mM) 30 min, followed by isoproterenol (5 mM) + propranol (0.1 mM) | 34 | 44 |
| Control                      | 34                    | 44                                           |
| Epinephrine (5 mM)           | 18                    | 55                                           |
| Epinephrine (3 mM)           | 18                    | 43                                           |
| Control                      | 18                    | 54                                           |

<sup>a</sup> Treatment was for 2 hr

Figures 5b, 5c, and 5d show the same cells at 1, 2, and 3 hr, respectively, after the medium has been changed. Over the 3-hr period, the melanocyte has changed slightly in shape, but the activity of both cells remains about the same.

Figure 6 was taken from a time-lapse film of cells treated with 10 mM DBcAMP. Figure 6a shows a melanocyte and keratinocyte before DBcAMP is added. After 1 hr in 10 mM DBcAMP, the cells are more active (Fig. 6b). A melanosome packet has pinched off. Little protuberances have formed on the melanocyte (arrows). Figures 6c and 6d show these cells 2 and 3 hr, respectively, after DBcAMP has been added. The melanosome packet has moved further into the keratinocyte; more protuberances have formed on the melanocyte. It is as if a number of melanosome packets are about to be pinched off; these packets, however, are not donated to the medium. Transfer has only been observed in the area of contact of a melanocyte with a keratinocyte. One hour after media without DBcAMP is added (Fig. 6e), cell activity has decreased, and the protuberances on the melanocyte are no longer observed.
**FIG. 4.** Effect of epinephrine on the rate of melanosome transfer from melanocytes to keratinocytes. Treatment in 3 mM epinephrine (●), 5 mM epinephrine (■), media only (○). Points are means ± SE for 18 cells.

**FIG. 5.** Melanocyte (M) and keratinocyte (K) from a culture of adult guinea pig ear epidermis. (a) untreated cells, (b) cells 1 hr after the media is changed, (c) the same cells 2 hr, and (d) 3 hr after the media is changed. Phase contrast, ×200.
Fig. 6. Response of a melanocyte (M) and keratinocyte (K) from an epidermal cell culture to DBcAMP. (a) untreated cells, (b) cells 1 hr after 10 mM DBcAMP is added. A melanosome packet (MP) has pinched off. Arrows indicate protuberances on the melanocyte, (c) the same cells 2 hr and (d) 3 hr after 10 mM DBcAMP has been added. More protuberances (→) are observed on the melanocyte, (e) cells 1 hr after media without DBcAMP is added. Phase contrast, ×248.

These same physiological effects are observed with cells treated with DBcAMP (8 mM) plus theophylline (5 mM), and with isoproterenol (5 mM). Treatment with propranolol (0.1 mM) inhibits changes in cell activity produced by isoproterenol. Epinephrine has no effect on cell activity.

Ultrastructurally, DBcAMP and theophylline do not appear to have any effect on the microfilament content of the melanocytes or keratinocytes. Figure 7 shows
FIG. 7. Longitudinal section through an untreated keratinocyte. A band of microfilaments (Mf) is present just beneath the cell membrane. Bundles of filaments (FB) and microtubules (Mt) are also present. ×16,200.

FIG. 8. Portion of a keratinocyte treated with 8 mM DBcAMP plus 5 mM theophylline for 3 hr. The cell appears the same as the control. The band of microfilaments (Mf), bundles of filaments (FB), and microtubules (Mt) are present. ×16,200.
a portion of an untreated keratinocyte. A band of 30–70 Å microfilaments is present just beneath the cell membrane. Bundles of 60–110 Å filaments as well as microtubules are also present in the cell. Figure 8 shows a portion of a keratinocyte treated with 8 mM DBcAMP plus 5 mM theophylline for 3 hr. The microfilament content is the same as that of the untreated cell. Untreated melanocytes also have a band of 30–70 Å microfilaments located just beneath the cell membrane and 40–110 Å filaments scattered throughout the cell (Fig. 9). Melanocytes treated with DBcAMP (10 mM for 2¾ hr) appear the same as the controls (Fig. 10).

DISCUSSION

Dibutyril cyclic AMP increases the rate of pigment donation from mammalian melanocytes to keratinocytes and also increases the number of melanocytes donating pigment. The cAMP phosphodiesterase inhibitor, theophylline, likewise increases the rate of melanosome transfer and enhances the effect of DBcAMP.

During the process of melanosome transfer in vitro, there is increased activity in the keratinocyte membrane adjacent to the area of contact with a melanocyte (1–3). Both DBcAMP and theophylline produce an increase in the ruffling of the keratinocyte cell membrane. They also increase the activity of the melanocytes. Numerous protuberances form on the melanocytes which look like packets of pigment which are about to be pinched off. These packets, however, are not donated to the medium. It appears that association of a keratinocyte with a melanocyte is necessary for melanosome transfer to occur.

A band of microfilaments just beneath the keratinocyte and melanocyte cell membrane has been implicated as being involved in the donation process (3). In keratinocytes, the microfilaments may be involved in undulation of the cell membrane, which in turn may be important in the transfer process. In melanocytes,
they may be involved in the pinching off of the melanosome packet. A correlation has been observed between disappearance of the band of microfilaments upon treatment with cytochalasin and cessation of cell-membrane activity and pigment donation (3).

The present studies show no apparent change in the microfilament content of melanocytes or keratinocytes treated with DBCAMP and theophylline. This may indicate that the effect of DBCAMP on microfilaments, if any, is indirect. This nucleotide may act through phosphorylation of microfilaments rather than through an increase in their number. It has been postulated that in some systems cAMP may exert its effect by stimulating phosphorylation of contractile proteins (17).

The mechanisms involved in pigment donation are still unclear. It appears that in vitro contact between melanocytes and keratinocytes is necessary for melanosome transfer. It is possible that there are specific sites, or receptors, on the keratinocyte and melanocyte cell surfaces which are stimulated when the cells come in contact. Contact alone, however, does not always lead to pigment donation. Some additional factor(s) seem to be involved.

The present work with \( \alpha \)- and \( \beta \)-adrenergic agonists indicates that stimulation of \( \beta \)-receptors enhances pigment donation. Treatment of melanocytes and keratinocytes with isoproterenol produces an increase in the activity of the melanocytes and keratinocytes, in the rate of pigment donation, and also in the number of cells donating pigments. This activity is inhibited by the \( \beta \)-adrenergic blocking agent, propranolol. Recent studies have shown that, in mammalian epidermis, \( \beta \)-adrenergic stimulation increases adenyl cyclase activity (18) and cAMP formation (19). It is possible that, in the present work, stimulation of \( \beta \)-receptors on melanocytes and keratinocytes leads to increased activity of adenyl cyclase with a consequent rise in cAMP levels. Increased levels of cAMP could then lead to stimulation of pigment donation.

An \( \alpha \)-adrenergic stimulator (4), epinephrine, had no effect on cell activity or melanosome transfer. Studies with rat epidermis have shown that norepinephrine produces little change in cAMP levels indicating that, in this case, epidermal \( \alpha \)-receptors are not involved in stimulation of cAMP synthesis (20). In some systems epinephrine also stimulates \( \beta \)-receptors (4). The fact that epinephrine had no effect on pigment donation may indicate that its \( \beta \)-stimulating action, if present, is not strong enough to enhance melanosome transfer or that its action on \( \alpha \)-receptors reduces the response to \( \beta \)-stimulation. Further work is needed to determine the role of these receptors.

The present studies indicate: (a) that cAMP may be an important factor in governing the rate of pigment donation from mammalian melanocytes to keratinocytes, and (b) that stimulation of \( \beta \)-adrenergic receptors mediates increases in the rate of melanosome transfer. It is possible that stimulation of these receptors leads to increased activity of adenyl cyclase with a consequent rise in cAMP levels. Increases in levels of this intracellular messenger could then lead to stimulation of pigment donation, possibly through activation of microfilaments. It is also possible that contact between melanocytes and keratinocytes stimulates these receptors, since there is increased activity in the area of contact, and melanocytes are only observed to donate pigment when associated with a keratinocyte.

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