IONTOPHORETIC RELEASE OF CYCLIC AMP AND DISPERSION OF MELANOSOMES WITHIN A SINGLE MELANOPHORE

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ABSTRACT

Selective dispersion of melanosomes was often observed after iontophoretic injection of cyclic adenosine monophosphate (AMP) from a glass microelectrode positioned in a target melanophore in frog skin (as viewed from above through a microscope), with other melanophores in the field serving as controls. Because the skin has orderly arrays of several types of closely spaced cells, it is probable that at times the microelectrode also impales cells other than melanophores. When cyclic AMP injection inside a cell resulted in dispersion of melanosomes from a perinuclear position into dendritic processes, the onset of dispersion was relatively rapid, in many cases <4 min (mean time of onset, 5.3 ± 2.9 [SD] min). A much slower dispersion (mean time of onset, 19.0 ± 5.0 min) of melanosomes was observed when the microelectrode was positioned adjacent to a melanophore, and much larger quantities of cyclic AMP were released. In addition, no changes were observed for injections of 5'-AMP or cyclic guanosine monophosphate (GMP) through electrodes positioned inside or adjacent to melanophores. Potential measurements showed that after impaling a cell, a constant transmembrane potential could often be recorded over many minutes, indicating that the membrane tends to seal around the microelectrode. The results indicate that cyclic AMP acts more rapidly on the inside of a cell than when applied outside a cell and allowed to diffuse through the plasma membrane. This study introduces a model system whereby the properties of the plasma membrane and melanocyte-stimulating hormone (MSH) receptors can be studied within a single target cell.

KEY WORDS melanophores · iontophoresis · cyclic AMP · intracellular injection · frog skin

Excluding electrical potentials, a relatively small number of parameters can be continuously measured in vitro in a single cell with addition of a hormone, a neural transmitter, or a substance such as cyclic adenosine monophosphate (AMP). Castillo and Katz (11, 12) first studied receptor properties using an injection through glass microelectrodes when they measured miniature end-plate potentials after iontophoretic injection of acetylcholine (ACh). They showed that the receptor for ACh on muscle cells had its binding site on the outer surface of the sarcolemma. More recently, Bloom and his co-workers have applied many dif-
ferent compounds to neurons via multibarreled electrodes, and have recorded the firing rate of single neurons in the cerebellum (8, 17, 28) and the hippocampus (27) of the rat. These latter experiments point out the wide variety of substances which can be iontophoretically released to delineate mechanisms of norepinephrine (NE)-mediated responses possibly involving cyclic AMP. The melanocyte-stimulating hormone (MSH)-mediated dispersion of melanosomes within melanophores (considered in this study) is another type of process than can be seen in a single cell. Moreover, dermal melanophores of *Rana pipiens* are several 100 µm in diameter (3), suggesting that intermediate steps leading to dispersion can be studied by iontophoretic injection of various substances.

Bagnara and Hadley (3) have reviewed studies on chromatophores and their color changes, including those pointing to a role for intracellular cyclic AMP as a second messenger in melanophores. Bitensky and Burstein (5) first showed that placing frog skins in a solution of $1 \times 10^{-7}-3.3 \times 10^{-8} \text{M}$ cyclic AMP leads to dispersion of melanosomes into the dendritic processes of dermal melanophores, thus imitating the action of MSH. Abe et al. (1, 2) later measured cyclic AMP concentrations within frog skin after application of MSH. 30 min after the addition of 5 µg of MSH, they found a significant increase of 0.5 nmol/g in pigmented dorsal skin, but not in pigment-free ventral skin. A similar effect of MSH on adenylate cyclase activity and cyclic AMP levels in mouse melanoma cells was described by Bitensky et al. (6) and Pawelek et al. (26). Varga et al. (32) subsequently postulated that the hormone acts on the melanoma cell's plasma membrane receptors because when MSH was covalently bound to Sepharose beads (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N.J.), preventing any possible diffusion through the melanoma cell membrane, the hormone on the beads could still elicit an increase in cyclic AMP levels. All these studies are consistent with the proposal that MSH acts to increase intracellular levels of cyclic AMP (3, 15).

Thus, frog skin melanophores appear to conform to the usual model for the action of polypeptide and protein hormones, and amines which utilize cyclic AMP as a second messenger, viz., the interaction of the hormone with plasma membrane receptors, followed by the activation of adenylyl cyclase, an increase in intracellular cyclic AMP concentrations, and a modulation by cyclic AMP of some biochemical event (e.g., protein kinase activation) to affect a change in the biological end point (in this case, dispersion of melanosomes). This model appears tenable despite the fact that even when a strip of frog skin is placed in a solution of cyclic AMP at concentrations many times greater than physiological levels, the darkening is still "quite slow and rather minimal" (3). It is generally assumed that the plasma membrane is relatively impermeable to cyclic AMP.

The following studies were initiated to test the current belief, as summarized above, that protein and polypeptide hormones and amines must exert their actions on target cells by first interacting with specific receptors on plasma membranes. Our ultimate objective is to determine whether these same hormones, when intracellularly injected, can exert their biological effects. The melanophore readily lends itself for such investigations, inasmuch as the response of a single cell can easily be observed under the microscope. The experiments to be described were conducted in order to characterize the properties of the system, and demonstrate that a known intracellular effector, 3',5'-cyclic AMP, can act when intracellularly injected, and also to demonstrate that the effect occurs more rapidly when it is restricted to the injected melanophore, rather than when the stimulant is extracellularly injected in close proximity to a melanophore.

**MATERIALS AND METHODS**

Over 350 *Rana pipiens pipiens* and *Rana pipiens berlandieri* (northern and southern frogs) were decapitated, and a circular patch of skin, 1 cm in diameter, was removed from the medial side of a hind limb, adjacent to the knee. The skin was placed in a holder and covered with frog Ringer's solution (NaCl, 6.5 g/liter; KCl, 0.14 g/liter; NaHCO₃, 0.20 g/liter; and CaCl₂, 0.12 g/liter). The holder was made with a Lucite base, and the skin was illuminated from below. In early experiments, a DC lamp (placed well below the stage to minimize radiated heat) was used, and in most experiments, a fiber optic cable was connected to an AC light source outside the shielded cage. The skin was viewed from above through a microscope, and a microelectrode was inserted into the skin at a 45° angle to the vertical axis of the microscope (Fig. 1). The skin was clamped along the border so that it lay over a surface in which a spiral groove was cut. The groove allowed the internal skin surface against the Lucite base to be bathed by the Ringer's solution, while providing a firm support for the skin. When the skin thickness was inappropriate for direct clamping, a porous disk was placed between the base and the frog skin. The arrange-
Figure 1 Sketch of mounting chamber and related apparatus. Fig. 1a, expanded view showing placement of frog skin over spiral groove cut in baseplate. The frog skin is held in place by a clamp, and is illuminated from below while viewed from above through a microscope. Fig. 1b, cross section showing orientation of multibarreled microelectrode with respect to frog skin. (Drawing not to scale.)

Data recorded here were for currents <200 nA for electrodes with an impedance which ranged from 20 to 200 MΩ, although most of the electrodes had a resistance which ranged from 50 to 150 MΩ. Occasionally, the tips of electrodes were broken so that a larger amount of a substance could be injected, and the current exceeded 200 nA for these electrodes.

Potentials were recorded with a Winston model S-857 preamplifier (Winston Electronics Co., San Francisco, Calif.), and displayed on a Tektronix 704N oscilloscope (Tektronix, Inc., Beaverton, Oreg.) and a Houston Instruments 3000 recorder (Houston Instrument, Div. of Bausch and Lomb, Austin, Tex.). The electrodes were advanced with a Kopf hydraulic microdrive (David Kopf Instruments, Tujunga, Calif.). The injection current was monitored by a Keithley 600B electrometer (Keithley Instruments, Inc., Cleveland, Ohio) in series with a lead to the injecting barrel.

Before or during the time the frog skin was on the Lucite base and impaled by electrodes, various compounds were added to the Ringer's solution bathing the skin. When *R. pipiens berlandieri* were used, and before mounting the skin on the base, a 2-μg/ml melatonin solution (Regis Chemical Co., Morton Grove, Ill.) was added to the Ringer's solution to obtain skins with melanosomes aggregated about the nucleus of each melanophore (paling the skin). To lighten the skin of *R. pipiens*, NE was added to the bathing solution. (Because the frogs were generally kept in white plastic trays under continuous 24-h lighting, the addition of skin-lightening agent was not always necessary.) A 2-mM solution of theophylline (Nutritional Biochemicals Corp., Cleveland, Ohio), a methylxanthine which inhibits the phosphodiesterase which hydrolyzes cyclic AMP, was added to the bathing solution. This theophylline concentration by it-
self was not sufficient to cause dispersion of melanosomes. At the conclusion of a series of electrode penetrations, agents causing dispersion (MSH) or contraction (NE in *R. pipiens pipiens*, melatonin in *R. pipiens berlandieri*) were often added to determine whether melanophores still responded to these agents.

Controls included injections of 0.1 M NaCl, Ringer's solution, 2.5 M KCl (because KCl-filled barrels give low liquid junction potentials and were used to measure resting potentials of cells), and dyes. Frog Ringer's solution, NaCl, and KCl were injected through a number of electrodes for 10- to 40-min intervals without observing melanosome dispersion. In most experiments KCl was not injected, and KCl-filled barrels were only used for measuring resting membrane potentials. In the control injections, no response was observed, although charged substances were readily transferred across the tips of electrodes as shown by the use of two dyes, acid fast green (Matheson, Coleman & Bell, Norwood, Ohio) (29, 30), and new methylene blue (Basic blue 24, Matheson, Coleman & Bell).

To determine whether the nucleotides used were iontophoretically released (10, 16, 25), microelectrodes were filled with one of the following (all obtained from New England Nuclear, Boston, Mass.): [G-3H]adenosine 3',5'-cyclic PO4 2- (NH4) salt, [G-3H]adenosine 5'-PO4 2- (NH4)2, or [G-3H]guanosine 3',5'-cyclic PO4 2- (NH4) salt, all solutions at pH 8. These three compounds were released from intact microelectrodes. For the labeled cyclic AMP, electrodes with broken tips (to allow passage of larger amounts) were found to pass 40-50 times as much as microelectrodes with intact tips. The net current through the microelectrode, as monitored by the Keithley electrometer, was a measure of the net movement of charged particles, and the use of labeled compounds showed that part of this current could be attributed to movement of the compound from the glass microelectrode into the frog skin. Therefore, when an electrode injection current was measured and no effect was observed, it was assumed that the agent (e.g., cyclic guanosine monophosphate [GMP]) was released from the electrode, but had no effect on the target cell.

RESULTS

Cyclic AMP Injection Within and Adjacent to Cells

After selecting a patch of skin having melanophores with melanosomes aggregated around the nuclei, a pattern shown in Fig. 2a and denoted M11 according to the Melanophore Index of Hogben and Slome (18), a microelectrode was advanced visually to a target cell (labeled A in Fig. 2a), and cyclic AMP was iontophoretically injected into the cell. Fig. 2b illustrates that after intracellular cyclic AMP injection, the melanosomes dispersed into a few of the dendritic branches of melanophore A, and the other cells in the immediate field remained unchanged. In this experiment, the microelectrode was then withdrawn and advanced toward a second melanophore, B, and again cyclic AMP injection resulted in dispersion (Fig. 2c). A summary of cellular responses of single melanophores (or of a small group of adjacent melanophores when cyclic AMP was injected outside a cell) is given in Table I.

![Figure 2](image)
Therefore, the melanophores throughout the field of the skin were identified even in the absence of gated stage) to a more dispersed stage. By comparing the whole field surrounding a target cell, cell population changes occurring over large areas of the skin were identified even in the absence of injection, and the experiment was terminated. Therefore, the melanophores throughout the field served as a control for population changes unrelated to an injection in either a single cell, or in a small region of skin containing a few melanophores.

Skins bathed with Ringer’s solution containing 2 mM theophylline to inhibit the breakdown of cyclic AMP, showed melanophores with the greatest dispersion after a cyclic AMP injection. The theophylline Ringer’s solution by itself produced no dispersion (Table I[d]). After the termination of cyclic AMP injection in skins to which theophylline was not added, the melanosomes continued to move radially for a period of several minutes, and then returned toward a perinuclear position. This reaggregation, several minutes after injection, is shown for cell A in Fig. 2c, where the melanosomes are closer to the nucleus than immediately after injection (Fig. 2b).

In several cases, one barrel of the electrode was used to record potentials after, as well as before injection, to determine whether or not the electrode remained within the cell during the injection period. For example, one impaled cell displayed a stable resting potential over a 1-min period. An injection current was then applied for 10 min, and the potential at the end of the injection period was almost the same as before the injection. The electrode was then removed from the cell, and the voltage returned to the base line found before impaling the cell. In this case, the melanosomes moved away from the nucleus when cyclic AMP was injected through the microelectrode sealed within the cell. In several cases, the transmembrane potential measured after injection was lower than before injection, and in other cases, the potential had returned to a level observed before the cell was impaled.

The electrical potential was also continuously measured through one barrel, to ensure that the electrode did not pierce the cell membrane when the microelectrode was placed adjacent to, but not in, a melanophore, as viewed from above. In all cases where the microelectrode was advanced close to a melanophore, but not close enough as to impale the melanophore, and cyclic AMP was injected from the intact microelectrode, there was no melanosome dispersion in the 10-min interval after cyclic AMP release (Table I[c]). Even when attempts were made to potentiate extracellular cyclic AMP effects by bathing the skin in theophylline-Ringer’s solution (Table I[d]), no dispersion was seen in the 10-min period after a 10-min injection. Therefore (in contrast to cases where the microelectrode was visually placed at the central area, although there was no occurrence of melanosome dispersion into dendritic processes without any change in the neighboring melanophores, 11 additional melanophores were observed in the 10-min period after a 10-min injection. The electrical potential was also continuously measured through one barrel, to ensure that the electrode did not pierce the cell membrane when the microelectrode was placed adjacent to, but not in, a melanophore, as viewed from above. In all cases where the microelectrode was advanced close to a melanophore, but not close enough as to impale the melanophore, and cyclic AMP was injected from the intact microelectrode, there was no melanosome dispersion in the 10-min interval after cyclic AMP release (Table I[c]). Even when attempts were made to potentiate extracellular cyclic AMP effects by bathing the skin in theophylline-Ringer’s solution (Table I[d]), no dispersion was seen in the 10-min period after a 10-min injection. Therefore (in contrast to cases where the microelectrode was visually placed at the center of the skin).
When the microelectrode was clearly outside any cell, as shown by concurrent electrical recordings, melanosome dispersion was not observed.

Because cyclic AMP, applied extracellularly in sufficient concentrations, results in melanosome dispersal (3), it appears that the absence of melanosome dispersal with the use of intact microelectrodes placed outside cells may have resulted from the injection of too small an amount of cyclic AMP for the skin volume in which it could be diluted. Therefore, the injection periods were increased to up to 1 h, but no clear and repeatable dispersion was observed. Consequently, the tips of several electrodes were broken, and again a voltage was applied to force a relatively large amount of cyclic AMP out of the electrode. After 11–26 min, several melanosomes in the field near the blunt tip of the broken electrode showed the onset of melanosome dispersion (Table I[e], Fig. 3).

![Figure 3a](image1.png)  
**Figure 3** Melanophore changes after injection of cyclic AMP outside cells, using microelectrodes with broken tips so that a larger amount could be injected. Fig. 3a shows field before injection. Fig. 3b shows dispersion of melanosomes in cells A and B after injection. × 200.

Cyclic GMP and 5'-AMP Injection Within and Adjacent to Cells

Using the same procedure reported above, 5'-AMP (at the same concentration, and adjusted to the same pH as the cyclic AMP used above) was injected within and adjacent to cells through intact microelectrodes and microelectrodes with broken tips. Theophylline was present in all experiments. Table I(f, g) indicates that there was no dispersion into dendrites for any injections, and no change occurred at all. Therefore, 5'-AMP does not result in the dispersion of melanosomes from the perinuclear area when injected inside or outside a melanophore.† These results imply that the dispersion

† At pH 7, cyclic AMP is a monovalent ion and 5'-AMP is divalent. Therefore, more cyclic AMP than 5'-AMP could be expected to be released for the same charge.
FIGURE 4 Histogram showing onset of melanosome dispersion for injection of cyclic AMP within cells (as viewed from above), and adjacent to cells. Intact microelectrodes were used for injection within cells, whereas microelectrodes with broken tips were used for injection outside cells.

noted for cyclic AMP injection cannot be attributed to a factor associated with injection, e.g., a change in membrane potential.

Cyclic GMP was also iontophoretically injected into melanophores (Table I[ti]), or was added to the solution used for bathing a frog skin. In 10 melanophores with aggregated melanosomes (MI1), no response was observed during or after delivery by the external circuit. This proved to be the case when, for a 5-min injection interval at 100 nA, approximately three times as many counts per minute (cpm) were ejected using cyclic AMP, as for 5'-AMP (equivalent concentrations of nucleotides with similar specific activities were used). To compensate for this difference in the amount of nucleotide released, 5'-AMP was injected at higher injection currents for a longer period of time, than cyclic AMP. In three cases, the onset of melanosome dispersion was noted in 2 min for injection currents as low as 10-20 nA for a 1-min injection period for pipettes filled with cyclic AMP. In contrast, using electrodes filled with 5'-AMP, we observed no dispersion during a 20-min period for injections into 20 cells at a current of 100 nA over a 15-min injection period. Therefore, 5'-AMP does not appear to evoke dispersion when released in much greater amounts than are necessary for cyclic AMP to cause dispersion. Additional support for this conclusion comes from experiments in which ejection of 5'-AMP for 60 min from microelectrodes with broken tips did not cause melanosomes to disperse from a perinuclear position.

Resting Membrane Potentials

The resting potentials of cells randomly impaled while advancing electrodes are summarized in Table II. The potentials of three cells which appeared to be melanophores when viewed from above, are shown in Fig. 5. The resistance of the electrodes ranged from 3 to 120 MΩ for the cells in Table II. These cells had stable resting potentials recorded for at least 30 s before the electrode was advanced or withdrawn. Recordings revealed a rapid drop in voltage as the microelectrode pierced the cell membrane. Although some cells could not be held for more than 30 s, others could be held for much longer periods. The cells in Table II include both melanophores and other cell types, and melanophores could not be distinguished on the basis of a characteristic resting potential. The fact that resting potentials could be recorded for long periods of time indicates that impaled cells tend to seal around the electrode.

DISCUSSION

The Plasma Membrane as a Barrier to Cyclic AMP Diffusion

The onset of melanosome dispersion was more

| Range of voltages | 30 mV or less | 30-40 mV | 40-50 mV | 50 mV or greater |
|------------------|-------------|---------|---------|-----------------|
| Number of cells  | 265         | 149     | 123     | 193             |

* Reference level was taken as 0 mV just outside the cell.
Resting potentials of three impaled dermal cells which appeared to be melanophores, when viewed from above. The electrode resistance was 20 MΩ.

rapid after injection of cyclic AMP within cells rather than outside cells, as shown by the histogram in Fig. 4. When the microelectrode tip was broken so that larger quantities of cyclic AMP could be extracellularly injected into the skin, a gradual dispersion of melanosomes was observed in a number of melanophores (Fig. 3), showing that sufficient quantities of cyclic AMP, extracellularly applied, eventually produce dispersion.

In 46 of 107 injections of cyclic AMP (Table I(a)), no change in melanosome dispersion was observed. Inasmuch as the skin has many cell layers and cell types, it is probable that the tip of the electrode was above or below the target cell, although it appeared to be near the nucleus of a melanophore. Typically, a microelectrode was oriented to impale the nuclear region of a target melanophore, and was advanced into the melanosomes aggregated around the nucleus. The M11 melanophores were clearly visible (Fig. 2), and usually spaced in a single layer for skin adjacent to the knee (other areas of the skin typically had multiple layers of melanophores or very few melanophores).

Moreover, in many cells, the electrode may not have remained within the cell during the entire injection period, or the cell membrane may not have been fully sealed around the electrode. Therefore, Table I(a,b) includes not only cases where the tip may have been above or below the melanophore, but also cases where the electrode may not have remained within a sealed cell. However, the fact that in 61 out of 107 cases, there was either a change from M11 to M12, or far more frequently, a further dispersion into dendritic processes, indicates that the relatively large size of melanophores makes them a fairly good target. Rather than impaling a few cells, determining that the membrane was sealed around the electrode, and injecting a fluorescent dye to permit histological identification of the impaled cell, the procedure used was to impale many cells and observe whether cyclic AMP would cause melanosome dispersion. The technique of sampling many cells, and scoring their responses into one of several categories (e.g., dispersal into dendritic processes, change from M11 to M12, or no change), followed the procedures of Bloom and his co-workers in their iontophoretic studies of neurons within the central nervous system (17, 27, 28).

Additional experiments were designed to determine whether the different rates of melanosome dispersion could be attributed to the injected cyclic AMP, or whether a complex of factors related to injection was involved in dispersion. The use of [3H]cyclic AMP showed that cyclic AMP was ejected from intact microelectrodes, and that larger quantities were released from microelectrodes whose tips were deliberately broken, as would be expected from the extensive studies on iontophoretic release of substances, including cyclic AMP (8, 16, 25). Intracellular injection of 5'-AMP or cyclic GMP did not result in radial movement of melanosomes (Table I(b)) under the...
same conditions used for injection of cyclic AMP, nor did injection of Ringer’s solution, NaCl, or KCl produce melanosome dispersion. Extracellular application of cyclic GMP had no effect in either paled or darkened skin, and extracellular release of cyclic AMP, from electrodes with broken tips, was required to elicit a clear effect. The fact that cyclic AMP causes melanosome dispersion when injected within a cell, and that higher amounts are required when applied outside a cell, together with the lack of response for 5'AMP, cyclic GMP, and KCl injections, indicates that this response was specific to cyclic AMP.

For many skins, theophylline was added to the Ringer’s solution (Table I) to inhibit the breakdown of cyclic AMP, and in those cells which responded to the injection, dispersion was more extensive than in the absence of theophylline. The response of cells to cyclic AMP was similar to that diagrammed in Fig. 6. The first stage of the response, a change from a small central aggregation to an enlarged circular area (from M11 to M12), can be interpreted as a movement away from the nucleus of the melanophore (3, 4). At stage M12, the melanosomes still lie near the bottom of the basket-shaped cells (Fig. 6b,e), and have not yet moved upward and radially into the dendrites of the cell. Further stages of the process involve such movement, but upon injection of cyclic AMP, it appeared that very often the melanosomes filled only a few dendrites. This selective or partial filling of a few dendritic processes (Figs. 2c, 6c) may reflect differences between injection of a bolus of cyclic AMP via a microelectrode within the cell, and the physiological increase in cyclic AMP concentration mediated by MSH.

The dispersal process is a complex one, and has been studied in a variety of laboratories in recent
years (7, 20, 23, 24). Nevertheless, the filling of dendritic branches on iontophoretic injection is similar to the pattern observed when the whole skin is bathed with MSH, or relatively high concentrations of cyclic AMP; the exception being that all dendrites are not always filled.

Therefore, the membrane appears to be an effective barrier to the easy diffusion of cyclic AMP, an observation consistent with data on melanophores (2, 3, 5) as well as on other tissues. Enzymes within the melanophore could also readily inactivate cyclic AMP diffusing at low rates through the plasma membrane. Equations developed for diffusion of compounds from microelectrodes (11, 25) indicate that cyclic AMP radially diffuses away from the electrode tip, once injected within a cell. It has not yet been determined why such diffusion does not always help to uniformly disperse melanosomes, but often results in partial or selective filling of dendritic branches.

Iontophoretic Injection, Resting Membrane Potentials, and Receptor Properties

Several investigators have sought to relate electrode current to the amount of cyclic AMP released from pipettes. Bloom et al. (9) have reviewed studies of cyclic nucleotides iontophoretically injected in the central nervous system, and they note that cyclic AMP has a transport number, \( t \), of approximately 0.05 assessed by injection of [\( ^{3}H \)] cyclic AMP into brain slices. Also, they find variation in the ability of different microelectrodes to deliver cyclic AMP. \( t \) is the ratio of the amount of a compound actually released, to that which would be released if all the charges were carried by the compound. For a \( t \) of 0.05, a current of 100 nA, and a 10-min injection period, the amount of cyclic AMP released from the electrode would be 31 pmol. The transport number of 0.05 for cyclic AMP is somewhat lower than the transport numbers for several other compounds (10, 16). In an independent study, Morrel et al. (25) have also measured amounts of cyclic AMP released by iontophoresis. Their results are not easily compared to those in our study, as they used solutions with lower concentrations of cyclic AMP, electrodes with lower resistances, and higher injection currents. From their data, one can estimate that approximately 11 pmol of cyclic AMP would be released over a 10-min interval, a current of 100 nA, and for electrodes filled with a \( 10^{-2} \) M solution of cyclic AMP. Our experimental procedures appear to be similar to those of Bloom and his co-workers, and therefore, as a first approximation, \( t \) can be assumed to have a value of 0.05.

Bloom and his co-workers (16, 17, 28) have used multibarreled microelectrodes for injection of drugs, and have further considered artifacts of this technique. The artifacts encountered for central nervous system complexes included (a) electrotonic effects of the injection current, (b) anesthetic effects of the drugs on neuron-firing rates, and (c) effects secondary to the pH of the solutions. With regard to the effect of injection currents, Bloom and his co-workers (17, 28) used balancing currents to avoid changing transmembrane potentials. In the experiments reported here, no correlation was found between melanosome configurations and resting potentials, and in a previous study on \( R. \ pipiens \), where the intent was to determine whether a correlation existed between membrane potentials and MSH-induced melanosome dispersion, no correlation was found either (21). Moreover, currents associated with 5'-AMP and cyclic AMP injection resulted in no dispersion. With regard to any long-lasting side effects which may have been produced by injected compounds, melanosomes still proved responsive to MSH and NE in \( R. \ pipiens \ pipiens \), and to MSH and melatonin in \( R. \ pipiens \ berlandieri \), at the close of experiments. Finally, Bloom and his co-workers maintained all drug solutions between pH 3 and 8, a range not directly affecting neurons. In control experiments on frog skin, injections at pH values below 9 had no dispersive effect, and all data in Table I were for solutions adjusted to a pH between 6 and 8.

Membrane potentials appeared relatively stable over a period of time (Fig. 5), and clearly distinct populations of dermal-cell resting potentials were not observed (Table II). For the measurement of resting potentials of cells, the reference level used was the potential just before impaling the cell. Frog skin melanophores lying in the dermis were separated by the basal lamina from cell layers in the epidermis. Potential profiles through the cells in the epidermis displayed a steplike increase from a reference level of 0 mV on the outside of the skin, up to +100 mV at the basal lamina (31, 33). Occasionally, impaled cells would not seal around the electrode, and no melanosome movement was evidenced even in these instances where the resting potential would exponentially fall to zero.

\(^{2}\) Occasionally, impaled cells would not seal around the electrode, and no melanosome movement was evidenced even in these instances where the resting potential would exponentially fall to zero.
The outer cell layers were joined by tight junctions (14) so that the surface cells within the epidermis formed effective compartments. The studies on potential profiles (13, 31, 33) were oriented toward detailing cellular potentials in relation to sodium ion transport across the amphibian skin. Whittembury (33), and Ussing and Windhager (31) presented data on potential profiles aligned with cellular layers by identifying recording sites with carmine deposits. These studies showed that if the skin is not short-circuited, active transport of sodium ions could establish a step-like potential shift across the skin, a factor considered in choosing reference levels for measuring resting potentials of cells within the skin. In the present study, the reference level was taken as 0 mV just outside a cell, and the resting potential of the cell was the negative deflection in potential, observed once the microelectrode had penetrated the cell membrane. With this choice of reference potential, no close range of potentials could be associated with the cells impaled, when a microelectrode was positioned within what appeared to be a melanophore when viewed through the microscope. Our findings are in contrast to those of Martin and Snell (21), who used the dorsal surface of the web between the second and third toes of R. pipiens, and reported two distinct groups of cells, one having a range of resting potentials from 35 to 40 mV (some type of dermal cell), and the other having resting potentials from 70 to 80 mV (which they denote as melanophores).

Preliminary results in which potentials were measured both before and after injections of cyclic AMP, together with the observation that resting potentials can be measured in cells for intervals exceeding 15 min (Fig. 5), suggest that additional melanophore experiments can be planned. The observation that the resting membrane potential was fairly stable in cells indicates that cells in frog skin will tend to seal around the glass electrode, preventing the leak of ions and charged particles from or into the cell. Experiments using iontophoretic injection, and requiring microelectrodes to remain sealed within the cell, are feasible. This is in contrast to the present study, which required only the initial placement of the electrode within the cell, but did not require the electrode to remain sealed within a melanophore.

Frog skin melanophores have been postulated to possess both MSH receptors and NE receptors (3), and their asymmetrical properties may be studied by using electrodes sealed within cells throughout the injection period, a study currently in progress. It builds on the findings of this study that iontophoretic injection of drugs within melanophores is possible, and that experimental data show that cyclic AMP is effective at lower concentrations and acts more rapidly when applied within the cell, rather than outside the cell.

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