Regulation of Melanophore Stimulating Hormone (MSH) Release

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Melanin pigmentation of the vertebrate integument is regulated by melanophore-stimulating hormone (MSH) which originates from the pars intermedia of the pituitary gland (1). Early experiments established that MSH release, as for other pituitary secretions, was under control by the hypothalamus (2). Similar to prolactin release (3), MSH secretion is under an inhibitory control by the hypothalamus (4). Hypothalamic lesions (5, 6) or ectopic transplantation (7) of the pituitary leads to hypertrophy of the pars intermedia and elevated levels of circulating MSH (8). Both a direct neuronal (adrenergic) inhibition (9) and a hypothalamic MSH release-inhibiting factor (MRIF, 10) may possibly regulate pars intermedia function. It is not yet clear whether there is a functional relationship between these two suggested mechanisms of pars intermedia regulation or whether one or both of these inhibitory mechanisms is operative in each of the vertebrate classes (11).

Whatever the nature of the hormone(s) or neurotransmitter(s) controlling MSH secretion, these first messengers (12) probably interact directly with the pars intermedia cells. An understanding of their mechanisms of action, as for other chemical messengers, is clearly an important goal of the physiologist interested in the control of melanin pigmentation. An attempt will be made here to discuss and evaluate the experimental data relating to the factors and mechanisms said to be involved in pars intermedia cell regulation.

MATERIALS AND METHODS

With the exception of the rat, both sexes of each animal species were utilized. Only female rats, however, were employed in the studies reported here. The frogs, Rana pipiens (80–190 g) and R. catesbeiana (180–210 g) and the toad, Bufo marinus (200–280 g) were purchased from Southwestern Scientific Supply Company (Tucson, AZ). Hamsters (90–115 g) were obtained from the Arizona Pet
Farm (Tucson, AZ). Sprague-Dawley-derived rats (140–210 g) were reared in our own facilities.

**Pituitary removal and incubation.** Pituitaries were removed from the animal immediately after decapitation by guillotine and placed in a Ca^{2+}-free medium for preincubation as described previously (13). Rat and hamster pituitaries were incubated at 32°C in a Krebs-Ringer bicarbonate (KRB), Trizma-buffered to pH 7.5. An amphibian Ringer Trizma-buffered to pH 7.1 was employed at 27°C for the frog and toad pars intermedia. All glands were incubated in a Dubnoff metabolic shaker under a 95% O₂, 5% CO₂ atmosphere. Incubations were of 2-, 4-, 6-, or 8-hr duration.

**Ionic and related pharmacological studies.** Ion-deficient media (Ca^{2+}-free, K⁺-free) and media containing the cardiac glycoside, ouabain (Sigma Chemical Co.) were prepared as described previously (13). Cytochalasin B (Imperial Chemical Industries) was dissolved in dimethyl sulfoxide solution (10 mg cytochalasin B/ml DMSO) and brought to its final concentration by an addition of the appropriate Ringer. Colchicine (Sigma Chemical Co.) and the Vinca alkaloids, vinblastine and vincristine sulfate (Lilly Research Laboratories), were used.

**Hypothalamic tissue extracts.** The hypothalamus (minus median eminence) was removed according to previously published methods (14) and placed in an ice bath in a ratio of three hypothalami per milliliter of Ringer and then homogenized. After centrifugation, the supernatant fraction was divided equally with one half boiled, at 100°C for 10 min) and the other half untreated. Both supernatant fractions (boiled, unboiled) were then used as incubation media. Experiments were also designed to test for possible enzymatic activity of the crude (unboiled) hypothalamic extract. Two groups (four glands each) of pituitaries were incubated in control Ringer to allow for a normal release of MSH. After a normal 2-hr incubation period, the incubation medium of both groups was pooled and subsequently divided into two 4-ml aliquots. One aliquot served as a control while the other was used to homogenize freshly removed hypothalamic tissue. Both aliquots were then placed in the metabolic shaker for a second 2-hr incubation and then bioassayed. In addition, acid extracts were prepared by removing the hypothalamic tissue and placing them directly into 1 ml of 0.1 N HCl followed by homogenization and centrifugation. The supernatant fluid was then neutralized to pH 7.1 by the addition of Trizma-buffered Ringer and used as an incubation medium.

**Peptides.** Highly purified synthetic tocinoic acid

\[
(L\text{-Cys-L-Tyr-L-Ile-L-Gln-L-Asn-L-Cys-OH})
\]

and its amide, tocinamide

\[
(L\text{-Cys-L-Tyr-L-Ile-L-Gln-L-Asn-L-Cys-NH}_2)
\]

were prepared by published methods (15, 16) as was the crystalline tripeptide, L-Pro-L-Leu-Gly-NH₂ (17). These structures were then lyophilized with lactose as carrier. Fresh solutions of these peptides were prepared on the day of testing.

**Bioassay.** The amount of MSH activity in the media following pituitary incubation was determined by the *in vitro* frog-skin bioassay for MSH as previously described (13) which utilized the photoelectric reflectance method of Shizume *et al.* (18) and Wright and Lerner (19). The media were usually diluted with amphibian Ringer to give final concentrations of one pituitary secretion per 200 or 400 ml
of Ringer (whatever concentration gave a submaximal skin-darkening response). The values reported represent the mean (±SE) darkening response of eight or more frog skins used for each bioassay. Since, in addition to MSH, some experimental media contained either EDTA, ouabain, etc., it was necessary, when possible, to add a similar amount of these agents to the control solutions to be bioassayed.

Statistics. The Student $t$ test for paired observations was used throughout to determine significance.

RESULTS

Melanophore-stimulating hormone (MSH) release is normally under an inhibitory control by the hypothalamus and there is a spontaneous release of the hormone into the medium from the isolated pituitary (rat, mouse (13), hamster (20), or pars intermedia (frogs, Rana pipiens, Rana catesbeiana; toad, Bufo marinus (20). This in vitro release of MSH can be reversibly inhibited by the removal of either calcium or potassium ions from the medium (Fig 1A, B). A Ca$^{2+}$ requirement for hormone release was demonstrated for all species studied (including the lizard Anolis carolinensis, 13) as was the K$^+$ ion (except in the mouse). These latter results using K$^+$-free media suggested the possible role of a Na$^+$–K$^+$ pump (active transport) mechanism in the release of MSH. This was confirmed by the

![Figure 1](image-url)

**Fig. 1.** Frog (R. pipiens) pars intermedia were incubated for 2 hr in a Ca$^{2+}$-free medium plus EDTA (A), a K$^+$-deficient medium (B), or in the cardiac glycoside, ouabain, at 10$^{-4}$ g/ml (C). In all cases, there was a near-total inhibition of MSH release compared to controls. A total of 36 pituitaries (four per experimental group) were utilized. MSH secretion was inhibited by cytochalasin B (5 × 10$^{-4}$ g/ml) but not by colchicine (10$^{-4}$ g/ml) by 6-hr incubation in the presence of these agents (D). Each value represents the mean (±SE) darkening response of eight frog skins to the MSH contained in the incubation media. $P$ values are noted above the bars where inhibition of hormone release compared to the control was significant.
use of the cardiac glycoside, ouabain, to further test this hypothesis. Ouabain (and the related glycoside, strophanthin K) strongly inhibited (Fig. 1C), reversibly and in a dose-related manner (13), the release of MSH from both mammalian and amphibian pituitaries. It is of interest that both a K+-free medium and ouabain failed to inhibit MSH release from the mouse pituitary, and this is apparently consistent with other investigations using cardiac glycosides on this rodent.

Both microtubules and microfilaments have been linked with some secretory processes (21–23). Certain so-called microtubular-specific (colchicine) and microfilament-specific (cytochalasin B) disruptive agents were used to test their possible effects on MSH secretion. Incubation of frog pituitaries in low levels (5 × 10⁻⁸ g/ml) of cytochalasin B resulted in strong inhibition of MSH release (Fig. 1D). The specificity of this blockade to hormone release was demonstrated by the failure of colchicine (Fig. 1D) or Vinca alkaloids (vinblastine, vincristine [24]) to similarly inhibit MSH release even at high (10⁻³ g/ml) concentrations. Whether these results implicate a role for microfilaments (rather than microtubules) in the mechanism of acute release of MSH remains to be determined. Recent evidence (25) from other cellular systems links the effects of cytochalasin to transmembrane transport mechanisms.

**Fig. 2.** *Rana pipiens* pars intermedia were incubated for 2 hr in crude hypothalamic extracts (A), in hypothalamic extracts which had been boiled (B), or in acid extracts of the hypothalamus (C). Only the crude-extract preparation produced a decrease in MSH activity in the medium (A). Next, the incubation medium taken after a normal 2-hr pituitary release of MSH was divided into two equal volumes, one of which was used to homogenate hypothalamic tissue (see Methods for details). Both preparations were then returned to the incubator for another 2-hr period and then bioassayed. Again, the medium with crude extract (D) showed a dramatic drop in MSH activity, whereas this loss of MSH activity was not evident in the boiled extract (E). These results strongly suggest MSH degradation by enzymatic action of the crude brain extract (A,D) rather than an inhibition of hormone release. A total of 40 pars intermedia, four per experimental group, were utilized. Each bar represents the mean (±SE) darkening response of eight frog skins to the MSH in the incubation medium under each experimental condition.
TABLE 1

| Incubation time (hr) | Percent darkening | Percent inhibition of release | P  |
|----------------------|-------------------|-----------------------------|----|
|                      | pH 7.1            | pH 6.9                      |    |
| 2                    | 33 ± 1.9          | 20 ± 1.5                    | 39% | <0.001 |
| 4                    | 36 ± 2.9          | 18 ± 1.8                    | 50% | <0.001 |
| 4                    | 39 ± 3.5          | 28 ± 3.8                    | 28% | <0.05  |
| 6                    | 37 ± 3.2          | 24 ± 2.7                    | 35% | <0.01  |

Amphibian Ringer was Trizma buffered to pH 7.1 and 6.9 and the stability of the pH was monitored throughout the duration of each incubation. Four R. pipiens pars intermedia were incubated under each experimental condition. Results are reported as the mean (± SE) darkening response of eight frog skins to the MSH released into the incubation medium under the two conditions of pH.

Previous in vitro results (14, 20, 26, 27) showed that incubation of pituitaries in hypothalamic extracts resulted in decreased amounts of bioassayable MSH (Fig. 2A). Unfortunately, these past studies (with few exceptions, 28) failed to rule out other possible factors that might contribute to decreased amounts of MSH in the incubation media. Neither boiled nor acid-extracted hypothalami significantly decreased the amount of bioassayable MSH in the media (Fig. 2B, C). These results strongly suggested an enzymatic breakdown of released MSH. Further evidence for this was provided by the observation (Fig. 2D, E) that crude (unboiled) hypothalamic extracts destroyed the MSH activity of added MSH, a result which was not obtained by similar incubations with boiled extracts. These results do not, however, rule out the possibility that boiling or acid extraction also destroy the activity of a postulated melanophore-stimulating hormone release-inhibiting factor (MRIF). These results, however, do stand in contrast to those of Kastin (28) who has reported (from one in vitro study) that boiled extracts still inhibit MSH release under conditions of long-term incubation.

Various procedures have been undertaken in our laboratory to determine the optimal in vitro conditions for the study of MSH release. Lowering of the pH of amphibian Ringer from pH 7.1 to 6.9 produces a considerable drop in bioassayable MSH (Table 1) from the frog pars intermedia. Extended incubations (8-hr) of pituitaries in Ca²⁺-free media or in hypothalamic extracts result in an elevation of MSH within homogenates of frog pars intermedia (Fig. 3). These data possibly suggest that factors which cause a decreased release of MSH result in a higher level of MSH within pars intermedia cells. In the present experiment (Fig. 3), although the control and Ca²⁺-free media maintained a stable pH of 7.1, the pH of the hypothalamic extract continually dropped to a pH of 7.0 or slightly below. These results using extracts do not, therefore, allow one to determine unequivocally whether the elevation of pituitary MSH results from a lowered pH (as in Table 1) or the possible existence of hypothalamic MRIF.

Recent studies, both in vivo and in vitro, have provided evidence that the side chain of oxytocin, L-Pro-L-Leu-Gly-NH₂ is the MRIF (29). Other workers (30) isolating this same tripeptide, as well as a pentapeptide (Pro-His-Phe-Arg-Gly-NH₂) (31), from bovine hypothalami and using different in vivo methods have shown that both of these peptides possess MRIF activity (32). In a previously published study (14) employing the ring structure of oxytocin, tocinoic acid
REGULATION OF MSK RELEASE

Fig. 3. *R. pipiens* pars intermedia were incubated for 8 hr in a normal Ringer, in the Ca²⁺-free EDTA Ringer, and in crude hypothalamic extracts. Glands were transferred to fresh media or extract after 4-hr incubation. After another 4 hr of incubation, the pituitaries were removed, rinsed, and homogenized in normal Ringer. Each homogenate was then diluted (by the additional Ringer) to the concentrations noted and then bioassayed. Each bar represents the mean (±SE) darkening response of eight frog skins to the MSH activity of the pituitary homogenates at the various dilutions indicated. Glands incubated in a Ca²⁺-free medium and in the crude hypothalamic extract showed higher MSH activity than did pituitaries incubated in normal Ringer. P values are indicated above the bars where a significant increase in pituitary MSH activity over controls occurred.

(L-Cys-L-Tyr-L-Ile-L-Gln-L-Asn-L-Cys-OH), a potent inhibition of MSH release was shown in both the rat (Fig. 4A) and the toad, *B. marinus* (Fig. 4C). The frog *R. pipiens*, however, was found to be refractory to this neurohypophyseal ring compound (Fig. 4B) while at the same time showing a high sensitivity to a calcium-deficient medium.

In all species studied, with the exception of the frog *R. pipiens* as previously indicated (20), we were also able to demonstrate an inhibition of MSH release (Fig. 5) with tocinamide, the amide of tocinico acid. However, when utilizing the synthetic tripeptide (suggested by others to be the MRIF [29, 30]) no such inhibition of MSH release could be shown (Fig. 5) in any of the mammals or amphibians which had evidenced such a high sensitivity to the oxytocin ring compounds (tocinico acid, tocinamide). Again, MSH release from pituitaries of *Rana pipiens* (suggested by the in vivo methods of other workers [32], to be sensitive to this peptide) was not inhibited (Fig. 5) by the synthetic tripeptide.

DISCUSSION

Some of the earliest endocrine experiments clearly established (33–36) that the pituitary gland was in some way involved in the control of integumental melanin
pigmentation. Other investigations soon demonstrated that integumental pigment cells were controlled by a hormone of pituitary origin (37) which was shown (38) to be specifically localized within the pars intermedia. This melanophore (melanocyte)-stimulating hormone (MSH) can affect skin pigmentation in all vertebrate groups from cyclostomes to man (except possibly that of birds [39]). The release of MSH is normally regulated (1) by environmental cues such as temperature, humidity, and light, the latter cue being received through the eyes (and possibly the pineal [40, 41]). This information conveyed through neuronal and synaptic transmission is ultimately received by the hypothalamus. How the hypothalamus in turn regulates the release of pituitary MSH is presently the concern of many investigators.

Etkin showed that MSH release was no longer inhibited when pituitaries were transplanted to an ectopic site (2). Pituitary transplantation or lesioning (5) of the hypothalamus results in hypertrophy of the pars intermedia (6, 7) which is reflected in an increase in melanin pigmentation of the skin, the latter apparently resulting from increased circulating levels of plasma MSH.

Etkin suggested that inhibition of MSH release was regulated by neurosecretory neurons which directly penetrate from the hypothalamus into the substance of the pars intermedia (42). Electrophysiological studies (40, 41) as well as brain injections of neurotransmitters (9) have implicated both acetylcholine and a catecholamine, most probably norepinephrine, in the control of MSH release. An adrenergic
FIG. 5. Tocinamide inhibited the in vitro release of MSH from the pituitaries of the animals noted except the frog R. pipiens. The tripeptide, L-Pro-L-Leu-Gly-NH₂, was without such inhibitory action of MSH release in any of the species studied. Each bar represents the MSH released from four pituitaries. Values represent the mean (±SE) darkening response of eight frog skins to the MSH released under each experimental condition. Incubations were of 4-hr duration. The P values are noted above the bars where an inhibitory activity was significant. All concentrations of tocinamide or the tripeptide are in grams per milliliter.

plexus (43) has been visualized by histological means within the pars intermedia of a number of vertebrates (including that of mammals; see 39, for references). Ultrastructural studies have described the presence of both adrenergic and neurosecretory neurons within the vertebrate pars intermedia (44, 45). The individual and integrated contributions of neurosecretory, adrenergic, and cholinergic neurons in MSH release control are yet to be precisely defined. The possible involvement of the pineal and a pineal hormone in the regulation of MSH release (40, 46, 47) further complicates the picture.

In addition to a direct neuronal influence on pars intermedia cell release of MSH, hypothalamic inhibiting and releasing factors are implicated in the regulation of MSH release. Extracts of the hypothalamus are reported (10, 48) to inhibit MSH release. The presence of an MSH-releasing factor (MRF) in the hypothalamus in addition to an inhibiting factor (MRIF) has been reported (49–51). More recently, there have been several publications on the structural nature of these purported hypothalamic factors (29–31, 51, 52).

As already pointed out in this paper and elsewhere (20), tocinic acid and especially tocinamide are quite active in inhibiting MSH release in vitro from pituitaries of the mammals studied (rats and hamsters) and some of the amphibians studied (Bufo marinus, Rana catesbeiana), but are not active in others (Rana pipiens). Thus, no claim were made that these peptides are the physiological MRIF. On the other hand, based on in vitro and in vivo experiments (29, 30), it was suggested that L-Pro-L-Leu-Gly-NH₂, the side peptide from oxytocin, is MRIF. The same peptide, isolated from bovine hypothalamic extracts and based on a different in vivo assay (32), was suggested to be MRIF. Other attempts,
however, to show MRIF-like activity for L-Pro-L-Leu-Gly-NH₂ in a number of different animals and assays (14, 20, 53) were unsuccessful as no MSH-release inhibition could be observed. To further complicate the issue a report has appeared (51) that Cys-Tyr-Ile-Gln-Asn-OH, a pentapeptide portion from the ring of oxytocin, stimulates release of MSH in vivo. Furthermore, a second peptide, Pro-His-Phe-Arg-Gly-NH₂, has been isolated from bovine hypothalamic extracts and is said to possess some MRIF-like activity (31). These various reports raise a number of important questions: (1) Are any of these peptides the real MRIF? (2) What is the physiological and pharmacological significance of these results? (3) How is MSH controlled by the hypothalamus? These interrelated questions will now be discussed.

First of all, we do not believe that there is conclusive evidence that any of the peptides mentioned in the preceding paragraph correspond to the natural MRIF(s). Tocinoic acid and tocinamide, peptides which are remarkably effective and specific inhibitors of MSH release in vivo in mammals, have variable or no inhibitory activity in amphibians where a bona fide MRIF might be anticipated to be most active (since frogs, for example, rapidly change color in response to MSH). Furthermore, these peptides have neither been searched for nor found in hypothalamic extracts. Most interesting relative to the structural nature of hypothalamic peptides is the recent report that pressinoic acid (the ring of vasopressin) has “potent corticotrophin-releasing activity” (54).

On the other hand, although L-Pro-L-Leu-Gly-NH₂ has been found in hypothalamic extracts, this is not surprising since the most probable part of oxytocin that might be expected to be found in hypothalamic extracts would be L-Pro-L-Leu-Gly-NH₂, a peptide which would be relatively stable to most of the common exopeptidases. In any case, we believe that the in vivo assay methods which have been reported to measure MRIF-like activity of hypothalamic extracts and the tripeptide are inadequate and to some extent mutually contradictory. Furthermore, we have been unable to repeat (14, 20) the in vitro activity reported (29) for the tripeptide. In the assay method of Celis et al. (29), depletion of pituitary MSH after treatment with hypothalamic extracts is taken to indicate the presence of an MRF. Inhibition of pituitary MSH depletion either by an extract itself or by an added substance (such as the tripeptide) is taken as a measure of MRIF activity. If the MSH content of the pituitary is the same as controls (i.e., no change in MSH content), this is interpreted as an inhibition of MSH release, or more precisely as an inhibition of the releasing factor (MRF) of MSH. This is in contradiction to the results of Kastin et al., (48) who reported that hypothalamic extracts which have MRIF activity increase pituitary MSH levels as a result of “direct” inhibition of MSH release. In other words, in somewhat simplified terms, one idea suggests that no change in pituitary MSH content is evidence for MRIF because an MRF is blocked, and another suggests that a raise in pituitary MSH is evidence for MRIF because normal MSH release is blocked. Which is valid? Is either valid?

It appears that the direct pituitary (frog) application assay (32) was developed by Kastin et al. because L-Pro-L-Leu-Gly-NH₂ was inactive in the other in vivo (rat) assays (the same wherein hypothalamic extracts were reported (48), to be active because of the presence of MRIF). It is puzzling that this tripeptide which has been nominated as MRIF should not work in the same assay system wherein an MRIF was first postulated by these same investigators. Since it is said that serum (frog) inactivates the tripeptide (in doses as high as 10 mg) it seems surprising
to us that these workers could report (47) the “presence of increased amounts” of L-Pro-L-Leu-Gly-NH₂ in rat plasma. (To our knowledge this would be one of the few reports of a hypothalamic factor being detectable in plasma). Celis et al. (29) appear to have no problem in measuring MRIF activity of the tripeptide in the rat in vivo (or in vitro). The results using the direct pituitary application assay are certainly not impressive since changes in melanophore index are minimal in response to the tripeptide. Also, the melanocyte indices that have been reported (e.g., 3.76 ± 0.07) by these workers are impossible to obtain by any presently known visual or objective methods (1).

Our view of the “positive” MRIF-like activities of various peptides in our own and other laboratories is that they probably represent the effects of poorly understood changes in the microenvironment which may or may not be of normal physiological significance (55). It would seem that a more readily interpreted in vivo assay for MSH release is needed, preferably one in which changes in MSH levels could be measured in the blood of an animal which was minimally manipulated surgically. In the in vitro assay, much more information about microenvironmental effects is clearly needed before one can be reasonably certain that the measured effect on MSH release is a direct result of the substance tested and not an indirect effect resulting from a change in the microenvironment. For example, Kastin et al. have reported (56) a “mass action-type direct feedback control of pituitary release” of MSH. Under similar and related experimental conditions we have been unable to support this “mini” feedback hypothesis; indeed, we have obtained essentially opposite results (55). Also, apparently a number of investigators (including ourselves [14, 20]) may have been misled by the use of crude hypothalamic extracts. The so-called MRIF activity of these extracts is possibly due to nothing more than a decreased concentration of MSH resulting from enzymatic degradation of the hormone. Our results using either boiled or acid-extracted hypothalamic extracts in vitro, fail to provide any support for the existence of a hypothalamic MRIF or (MRF). In fact, it is now being suggested that the activity of hypothalamic extracts with regard to their effects on MSH release may depend upon the relative amounts of MRF and MRIF in the extracts which are affected by previous treatment of the particular extract (57).

Underlying all of these difficulties of interpretation is the nagging question of whether control of MSH release from the pars intermedia is under a neurosecretory control or an adrenergic control. Evidence is available in support of both interpretations, but it seems to us that neither control mechanism is firmly established. Certainly more work is needed to determine whether one or both are either singly or together involved. Basic to all of these questions is whether MSH secretion is under inhibitory control only or whether both inhibitory and release control mechanisms are important. The evidence for inhibitory control of MSH release appears to be firmly established from both the in vivo and in vitro data, but whether a releasing factor is also operative in the control of MSH release is still uncertain. To the best of our knowledge, only Taleisnik and co-workers have any strong evidence for such a control, and apparently other investigators have been unable to provide support for its existence.

Until these and other questions are acceptably answered theories about the nature of MRIF (whether a peptide or some other substance) will have to be acceptably tested. A disturbing aspect of these attempts to elucidate the structure(s) of these hypothalamic factors is the use of the word “hormone” rather than “fac-
"inhibit" in the designation of these hypothalamic substances before they have qualified for such identity by normal endocrinological rules.

The most promising studies on the control of MSH secretion relate to the cellular mechanisms involved in release of this hormone from the pars intermedia cell. The demonstration of a Ca\(^{2+}\) ion requirement for hormone release was not unexpected since nearly all cellular secretory events require Ca\(^{2+}\) (58) in what has been referred to as a "stimulus-secretion coupling" which has been likened to the "excitation-contraction coupling" in muscle (59). Most important was the demonstration of a K\(^{+}\) ion requirement for MSH release which had previously only been theorized (60) as a possibility for the mammotrophs of the pars distalis, which like pars intermedia cells are under an inhibitory influence by the hypothalamus. These results relative to the K\(^{+}\) ion suggested a possible role for a Na\(^{+}-K^{+}\) pump (active transport) system and this was (in our opinion) confirmed by the demonstration that ouabain (a cardiac glycoside) also inhibited MSH release. Of interest here is the recent report that the use of ouabain resulted in the release of ACTH and growth hormone from the rat pituitary (61). This result is consistent with earlier studies on other secretory systems (see 13, for references). Although such data appear at first contradictory, it must be emphasized here that they may actually provide important clues to the mechanisms involved in "stimulatory" versus "inhibitory" secretory systems. Ouabain may cause an increased influx of Na\(^{+}\) (and a possible sodium-dependent Ca\(^{2+}\) influx, 62) leading to membrane depolarization which would then provide the stimulus for hormone secretion (63). On the other hand, as suggested previously (13), inhibition of MSH release may result from cellular hyperpolarization of previously "spontaneously depolarized" (64) pars intermedia cells resulting from K\(^{+}\) efflux concomitant with ouabain-inhibition of K\(^{+}\) influx.

Our results using cytochalasin B are consistent with numerous other studies which have demonstrated that this drug is a potent inhibitor of cellular secretions (21, 23, 24), including the "cytocrine" (secretory) activity of epidermal melanocytes (65, 66). Whether these findings really implicate a role for microfilaments in hormone secretion remains to be shown. The failure to demonstrate "microtubular-microfilamentous structures" "connecting either the secretory granules with one another or the granule with the cell membrane" certainly need not imply "an absence of a relationship in the pars intermedia" (67) since, to our knowledge, only Lacy et al. (21) in his original observation on insulin-secreting cells has been so fortunate to make such an observation. Such an event is not likely to be easily captured in an electron micrograph (68).

Although there are many inconsistencies and unresolved problems concerning the control of MSH secretion (69), these latter studies on the cellular mechanisms regulating MSH release suggest that general answers relating to MSH and hormone secretion in general may be resolved in the near future.

**SUMMARY**

Synthetic tocinamide and tocinoic acid, ring structures of oxytocin, reversibly inhibit (at nanogram, or less, concentrations) the release of melanophore-stimulating hormone (MSH) from the rat and hamster pituitary in vitro. These peptides are less effective (on Bufo marinus and Rana catesbeiana) or totally without effect (on Rana pipiens) on MSH release from the isolated amphibian pars intermedia.
Oxytocin, lysine vasopressin, and ring structures of the vasopressins (pressinamide and pressinoic acid) are without effect of MSH release in the animals studied. The synthetic tripeptide side chain of oxytocin (L-Pro-L-Leu-Gly-NH₂) is devoid of in vitro MSH release inhibition in either the mammal or the frog.

Crude hypothalamic extracts from either the frog or the rat "appear" to reversibly inhibit in vitro MSH release. If, however, the extracts are heated to boiling or acid extracted, inhibition of MSH release is not observed, suggesting enzymatic or other reactions may be responsible for the loss of bioassayable MSH. The addition of MSH to crude hypothalamic extracts results in a loss of hormone activity. It is, therefore, unclear whether hypothalamic extracts provide evidence for the existence of a hypothalamic MSH release-inhibiting factor (MRIF). It remains for further work to establish whether the ring structures of the neurohypophysial hormones can be considered as possible candidates for a natural vertebrate MRIF.

Both calcium and potassium ions are necessary for MSH release in vitro. Release of the hormone is inhibited by ouabain and related cardiac glycosides suggesting that a Na⁺–K⁺ pump (active transport) system is involved in MSH release. Cytochalasin B, but not colchicine (or vinblastine and vincristine) is reversibly inhibitory to hormone release and suggests that a microfilament (rather than a microtubular) system may be involved in the mechanism of acute release of MSH. However, other actions of cytochalasin B on transport mechanisms or ionic fluxes may account for its inhibition of MSH release. The relationships between an active transport system, calcium ions, and a microfilament system in the control of MSH release by hypothalamic substances, neurohypophysial peptides, or related structures are still unclarified.

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