IDENTIFICATION OF MSH RELEASE-INHIBITING ELEMENTS IN THE NEUROINTERMEDIATE LOBE OF THE RAT

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SUMMARY

Neurointermediate lobes of rats comprise elements which, when excited in vitro, bring about an inhibition of the release of melanocyte stimulating hormone (MSH). Superfusion of neurointermediate lobes of intact donor rats with medium containing 45 mM K⁺ induced a stimulation of the release of oxytocin, arginine-vasopressin and dopamine (DA) and inhibited the release of MSH. Fluorescence histochemical observations and the results of release studies indicate that electrothermic lesions in the medio-basal hypothalamus induced a more rapid degeneration of dopaminergic than of peptidergic terminals in the neurointermediate lobe. Dopaminergic nerve terminals and the stimulated release of DA had vanished completely on the second day after these lesions, which coincided with the disappearance of K⁺-induced inhibition of MSH release.

Frontal hypothalamic deafferentations resulted in disappearance of peptidergic nerve terminals as evidenced by the development of diabetes insipidus and the strong decline of depolarization-induced release of oxytocin and vasopressin from neurointermediate lobes in vitro. In contrast, the dopaminergic plexus was left intact, as was the K⁺-induced stimulation of DA release and inhibition of MSH release.

We conclude that the K⁺-induced inhibition of MSH release is mediated by DA rather than by neuropeptides from terminals in the neurointermediate lobe. The results are in agreement with the proposed MSH release-inhibiting role of the dopaminergic tuberoinfundibular neurones.

INTRODUCTION

Since 1965 the identity of the mechanisms mediating the central control of MSH secretion has been the subject of many studies. Hypothalamic extracts were found to contain MSH release-stimulating and/or inhibiting factors, and it was suggested that
these factors are secreted into the portal blood in the median eminence. Although little is known on the local haemodynamics in the hypothalamo-hypophysial complex, the organization of the vascular bed makes it unlikely that factors secreted in the median eminence can reach the pars intermedia cells efficiently. Therefore, we have postulated that chemical signals involved in the control of MSH secretion are generated by elements located in the neurointermediate lobe itself.

The possibility that peptide factors control MSH release is supported by the presence of peptidergic nerve terminals in the pars intermedia of some mammalian species. However, the intermediate lobe of rats lacks peptidergic nerve terminals. Thus, if peptidergic factors are involved in the rat, such factors might be released by neurosecretory nerve terminals in the pars neuralis. In support of this possibility, Taleisnik et al. have found MSH release-stimulating and -inhibiting activity in the paraventricular-neurohypophyseal tract and the supraoptic nucleus, respectively. In addition, an MSH release-inhibiting factor has been chemically characterized as being identical to the 'tail-fragment' of oxytocin: Pro-Leu-Gly-NH$_2$. Furthermore, Celis has demonstrated the existence in the hypothalamus of enzymes which catalyze the formation of MSH release-stimulating and -inhibiting factors from neurohypophysial hormones. Although several workers, including ourselves, were not able to demonstrate MSH release-inhibiting effects of hypothalamic extracts and of synthetic Pro-Leu-Gly-NH$_2$ (cf. ref. 29), this does not exclude a possible peptidergic link in the control of MSH release.

In addition to neuropeptides, neurotransmitter substances present in the neurointermediate lobe might play a role in the control of MSH secretion. Electron microscopical and histochemical studies have shown that the pars intermedia of the rat, like that of most other vertebrates, contains a network of delicate nerve fibres making synaptic-like contacts with the MSH-producing cells. As demonstrated by Björklund et al., these fibres originate from dopaminergic neurones located in the rostral zone of the arcuate nucleus of the hypothalamus. Recently, we demonstrated that neurointermediate lobes of rats contain elements which, when stimulated in vitro, lead to inhibition of MSH release. We speculated that these elements were nerve terminals of dopaminergic neurones originating in the central nervous system (CNS). Such neurones could provide a means by which the CNS could control MSH secretion in vivo. The aim of the present study was to elucidate the nature of the MSH release-inhibiting elements in the neurointermediate lobes of the rat.

MATERIALS AND METHODS

Adult female rats of the Wistar strain (140–180 g body weight) were used. The animals were housed under a regimen in which the light period was from 07.00 to 21.00 h. Food and water were provided ad libitum.

Hypothalamic lesions

Electrothermic lesions were made in the mediobasal hypothalamus of ether anaesthetized rats as described earlier. The lesion included the median eminence, arcuate
nucleus, and parts of the ventromedial and premammillary nuclei and reached from 1 mm behind the optic chiasma to the mammillary bodies. Rats were sacrificed between 4 h and 32 days after surgery.

*Frontal hypothalamic deafferentations.* Each rat was anaesthetized with ether, and the head was fixed in a stereotaxic apparatus. An incision was made in the scalp through which an opening about 2 mm long was drilled in the midline of the skull. With the aid of a knife made of a sharpened stainless steel wire (Fig. 1), a semicircular frontal cut was made in the hypothalamus. After the operation, each rat was housed individually in a metabolic cage for one day, and water intake and urine production were measured. Only those rats which produced more than 50 ml of urine during the first 24 h after lesioning were used in further studies. According to histological examination, the semicircular lesion was positioned just rostral to the arcuate nucleus and excluded the supraoptic and paraventricular nuclei (Fig. 1). Animals in which the knife was not rotated served as sham-operated controls. Lesioned and sham-operated rats were killed by decapitation 7 or 14 days after surgery.

*Fluorescence histochemistry*

The formaldehyde-induced fluorescence (FIF) method was used for studying the localization of endogenous catecholamines in the pituitary gland. Whole pituitary glands were quenched into liquid nitrogen within 30 sec after decapitation. The glands were processed as described elsewhere in detail.28.

*Uptake of $[^3\text{H}]$dopamine.* Neurointermediate lobes were separated from anterior lobes and incubated under conditions which are known to label catecholaminergic nerve endings selectively. Briefly, lobes were preincubated at 37 °C in 5 ml of Krebs–Ringer bicarbonate buffer (KRB) containing $5 \times 10^{-4}$ M ascorbic acid and $10^{-9}$ M mialamide (an MAO inhibitor). After 15 min preincubation under an atmosphere of 95% oxygen and 5% carbon dioxide, 5 μCi $[^3\text{H}]$dopamine ($[^3\text{H}]$DA, final concentration: $5 \times 10^{-8}$ M) was added to the incubation fluid and the incubation was continued for another 20 min. After rinsing 3–6 neurointermediate lobes were transferred to each
chamber of a superfusion apparatus. As in studies with brain slices, the subsequent overflow of radiolabeled material was used as an index for the release of endogenous catecholamines.

**Superfusion of neurointermediate lobes.** Freshly isolated neurointermediate lobes or lobes preincubated with[^1H]DA were continuously superfused at 37 °C at a rate of 0.25 or 0.5 ml per min with oxygenated KRB medium. The effluent medium was collected in fractions of 5–20 min. After 40–50 min, the lobes were superfused for 15–30 min with KRB medium containing 45 mM KCl. The NaCl concentration was reduced appropriately in order to maintain iso-osmotic conditions. Thereafter, the superfusion was
Fig. 3. In vitro release of neurogenic material from neurointermediate lobes of rats with electrothermic lesions in the mediobasal hypothalamus. Lobes were preincubated in the presence of $[^3]H$ dopamine (5 x $10^{-8}$ M). Lobes of rats 24 h after sham-operation ---; 24 h postlesioning . . . . ; 48 h postlesioning ------; and 72 h postlesioning ●●●●●●●. K$^+$ indicates period of superfusion with medium containing 45 mM K$^+$. Continued with normal KRB for another 15–30 min. This method for examining release of $[^3]H$ DA from neurointermediate lobes is used routinely to study release of radiolabelled DA and other transmitter substances from brain slices and synaptosomes. It has been shown elsewhere$^{14,22}$ that K$^+$-induced release of tritium from tissue labelled previously by incubation with $[^3]H$ DA is Ca$^{2+}$-dependent and consists for the major part of unmetabolised $[^3]H$ DA.

**Determination of hormones.** MSH was measured in pituitary extracts and freshly collected superfusion medium samples using an in vitro assay with skin fragments of the lizard *Anolis carolinensis* as described previously$^{26}$. Synthetic α-MSH (Ciba-Geigy) was used as a reference standard. Oxytocin and vasopressin were measured by radioimmunoassays (for details see ref. 9). Cross-reaction of arginine-vasopressin and oxytocin was less than 0.1% in the heterologous assays. Standard curves were made in the presence of KRB medium.

**RESULTS**

**Effects of hypothalamic lesions on formaldehyde-induced fluorescence (FIF) of intra pituitary nerve fibres**

In histochemical preparations of pituitary glands of untreated rats varicose nerve fibres, generally appearing as small fluorescent dots, form a plexus in between the endocrine cells of the intermediate lobe (Fig. 2a). A similar network of delicate varicose fibres is present throughout the pars neuralis where accumulations of varicosities were found around the capillaries, particularly in the rostral part.

Frontal deafferentations of the hypothalamus did not induce marked changes in
fluorescence intensity or plexus density of the varicose fibres in the neural and intermediate lobe (Fig. 2e).

In rats with electrothermic lesions in the mediobasal hypothalamus FIF progressively accumulated in these fibres and after 8 h strongly fluorescent varicosities and large fluorescent dots were found throughout the pars intermedia and pars neuralis (Fig. 2b). However, one day after lesioning the previous piling-up of FIF had disappeared and the varicosities showed a fluorescence intensity comparable to that of sham-operated and intact rats (Fig. 2c). From the second postlesioning day we never observed varicosities displaying FIF, not even after pretreating the animals with nialamide (Fig. 2d), except for some sympathetic vasomotor fibres associated with arterioles penetrating the pars neuralis.

**Effects of hypothalamic lesions on the release of oxytocin, vasopressin and $[^3H]DA$ from isolated neurointermediate lobes in vitro**

As illustrated in Figs. 3 and 4, membrane depolarization (KRB containing 45 mM K$^+$) evoked a strong release of oxytocin, vasopressin and $[^3H]DA$ from neurointermediate lobes of sham-operated rats.

With regard to the release of $[^3H]DA$, neurointermediate lobes of rats with electrothermic lesions in the mediobasal hypothalamus for 24 h responded similar to those of sham-operated rats, but from the second postlesioning day no K$^+$-induced release
Fig. 5. In vitro release of MSH from neurointermediate lobes of rats with hypothalamic lesions. A: lobes of rats with electrotic lesions for 24 h ———; and for 48 h ————. B: lobes of rats with frontal hypothalamic deafferentations for 14 days ——— and sham-operated controls ————. K⁺ indicates period of superfusion with medium containing 45 mM K⁺. Data show results of representative experiments (see text).

could be observed. The changes in the release of oxytocin and vasopressin, however, followed a different time course. As illustrated in Fig. 3 the depolarization-induced release of oxytocin and vasopressin showed a gradual decline after lesioning. This coincided with a progressive decline in oxytocin and vasopressin content of the lobes.

In one experiment, we studied the release of [³H]DA, oxytocin and vasopressin by groups of neurointermediate lobes from rats two weeks after frontal hypothalamic deafferentation or sham-operation. As illustrated in Fig. 4 the K⁺-induced release of both neuropeptides had largely disappeared in the lesioned group which is consistent with a decrease in oxytocin and vasopressin content to 4.8 and 11.1 %, respectively, of that of the sham-operated controls. In contrast to the neuropeptides the K⁺-induced release of [³H]dopamine was hardly affected in the lesioned group.

Effects of hypothalamic lesions on the release of MSH from neurointermediate lobes in vitro.

After about 30 min of superfusion with normal KRB neurointermediate lobes of intact donor rats reached a stable secretion rate of about 2.5 ng (range: 0.9–4.2 in 29 experiments) per lobe per 10 min. Variation in basal MSH secretion of duplicate runs was usually small, but differences of up to 50 % were found occasionally. Despite the pronounced initial efflux, the total amount of MSH present in the effluent medium after 2 h of superfusion was rarely over 4 % of total MSH content in extracts of superfused lobes. In addition, in 3 experiments with two groups of 7–10 neurointermediate lobes each no differences (P > 0.1) were found between MSH content of lobes superfused for 2 h and their non-superfused controls. When neurointermediate lobes of untreated or
sham-operated rats, after reaching a stable MSH secretion rate, were superfused with a medium containing 45 mM K⁺ the rate of MSH secretion gradually fell to 46 ± 3% (mean ± S.E.M. of 29 experiments) of basal values within 30 min. Prolongation of the K⁺ stimulus did not induce further inhibition of MSH secretion, but hampered a prompt return to its basal level after termination of the K⁺ stimulus. As illustrated in Fig. 5B neurointermediate lobes of rats with frontal hypothalamic deafferentations for 14 days, showed a reversible decrease in basal MSH secretion rate to 42 ± 7% (mean ± S.E.M. of 6 experiments) in response to the K⁺ stimulus. No reproducible differences were found in basal MSH secretion rate between lobes of frontal hypothalamic deafferented and sham-operated rats. Neurointermediate lobes of rats with electrothermic lesions in the mediobasal hypothalamus for 1 or 2 days consistently showed a decrease of basal MSH efflux to 10–50% of their respective sham-operated controls. As reported earlier, 1 and 2 days after lesioning pituitary MSH content fell to about 25% of that of controls. In spite of this low MSH release neurointermediate lobes of rats with electrothermic lesions for 1 day still responded to 45 mM K⁺ with a usual transient suppression of MSH release (Fig. 5A). In contrast, we never observed a K⁺-induced inhibition of MSH release in any of the 5 experiments with lobes derived from rats on the second post-lesioning day.

DISCUSSION

Neurointermediate lobes of intact rats showed a basal release rate in the order of 15 ng MSH per hour, which reflects 0.5–1.0% of pituitary MSH content. Although such a figure seems to be in agreement with basal circulating MSH levels in the rat (100–250 pg/ml)⁴,⁶,¹⁸,¹⁹, it is surprisingly low in comparison to data obtained from frog pituitaries in vitro. This is more surprising since the release of MSH, which is generally accepted to be under a tonic inhibition of the CNS, may be expected to increase strongly when disconnected from its central control. Therefore, the question arises whether the in vitro release of MSH was still continuously suppressed by some MSH release-inhibiting factor in the preparation. Although substances from central neuronal origin (e.g. dopamine and neuropeptides, see Fig. 3) were spontaneously released into the medium, it is unlikely that they would interfere with basal MSH release, since neurointermediate lobes of rats with chronic lesions in the mediobasal hypothalamus, leading to degeneration of nerve fibres in the pituitary, showed a similar spontaneous secretion rate of MSH.²⁷

Assuming that the spontaneous release of MSH indeed reflects uninhibited secretion some degree of persistent membrane depolarization of pars intermedia cells might be proposed. If so, this might also explain our earlier finding that K⁺ depolarization did not affect the secretion rate of MSH from pars intermedia cells directly.²⁷ Similarly, the in vitro release of prolactin, another pituitary hormone which is subject to inhibitory control by the CNS, was not affected by K⁺ depolarization.²⁷,²⁸ Accordingly, the K⁺-induced inhibition of MSH release observed in neurointermediate lobes of intact donor rats was suggested to be mediated by depolarization-induced secretion of some MSH release-inhibiting factor within the preparation.²⁷ Since peptidergic and dopaminergic
nerve terminals are the most likely candidates we have attempted to discriminate between them by correlating the stimulated release of these neurogenic substances to changes in MSH release. In view of the non-selective nature of the stimulus used in these experiments and the relationship between the proposed MSH release modulating factors and neurohypophysial hormones, the release of oxytocin and vasopressin was considered as an index for the release of neurogenic peptides in general.

After making electrothermic lesions in the mediobasal hypothalamus, a striking difference was found in the time course of degeneration of peptidergic and dopaminergic nerve terminals. The peptidergic fibres showed a slow progressive degeneration over a period of about 5 days as indicated by: (a) characteristic triphasic changes in water balance after lesioning (cf. refs. 13 and 30); (b) gradual loss of aldehyde fuchsin stainable neurosecretory material in histological preparation which is complete after 5 days; and (c) gradual reduction in size of the K$^+$-induced release of these neuropeptides which is still continuing after 3 days (Fig. 3).

Histochemically, accumulations of catecholamines were detected shortly after lesioning (Fig. 2b). Indeed, axotomy has been reported to induce a transient increase in the content of catecholamines in terminal axons which, in contrast to neuropeptides, can be synthesized within the nerve terminal itself. One day after lesioning the histochemical characteristics and K$^+$-induced release of $[^3]$H]DA were similar to those of lobes of sham-operated rats. On the second postlesioning day, however, the dopaminergic fibres seemed to have degenerated completely, as indicated by both histochemical (Fig. 2d) and release studies (Fig. 3). Interestingly, this sudden degeneration of dopaminergic fibres in neurointermediate lobes coincided with the disappearance of the K$^+$-induced inhibition of MSH release which is present on the first, but absent on the second postlesioning day (Fig. 5).

After frontal hypothalamic deafferentation peptidergic fibres running to the pars neuralis were interrupted selectively. In agreement with this the animals suffered from diabetes insipidus and their neurointermediate lobes contained very little oxytocin and vasopressin and no aldehyde fuchsin-positive staining material. Moreover, only a small K$^+$-evoked release of neurogenic peptides was found. In contrast, the K$^+$-induced release of dopamine was comparable to that of sham-lesioned rats, which is compatible with fluorescence microscopical observations showing a normal appearance of the dopaminergic innervation of the pars intermedia (Fig. 2). Furthermore, 45 mM K$^+$ evoked a reversible inhibition of MSH secretion from neurointermediate lobes of rats with frontal hypothalamic deafferentations which was identical to that of sham-operated controls.

The present results show that the release of neurogenic peptides evoked by K$^+$-depolarization did not correlate with the K$^+$-induced inhibition of MSH secretion. On the other hand, K$^+$-induced inhibition of MSH release was always associated with a stimulation of the release of dopamine. In addition, after pretreatment of rats with reserpine resulting in a depletion of neuronal dopamine stores in the pars intermedia (see Fig. 2f), the response of MSH to 45 mM K$^+$ disappeared. Recently, we found that the inhibiting effect of K$^+$ on MSH release could be blocked by the dopamine-receptor antagonists haloperidol and pimozide and mimicked by dopamine and apo-
morphine (Tilders et al., in preparation). We conclude, therefore, that 45 mM K+ stimulated the release of dopamine from nerve terminals in the neurointermediate lobe which, via an interaction with dopamine-receptors, resulted in an inhibition of MSH release. Thus the present data confirm our earlier hypothesis that the dopaminergic tuberohypophysial neurones mediate the inhibitory effect of the CNS on MSH secretion.

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