bound IgG in a quantum fashion similar to that induced by TPA.

The use of flow cytometry to analyze intracellular metabolic processes promises to yield additional comparative information about the mechanism of cell stimulation by a wide variety of agonists. The technique requires only that the agonist produce a discernible change in either the intrinsic cell fluorescence properties or in fluorogenic substrates added to the cell *(22, 23)*. Changes in intracellular *p*H during metabolic events can also be monitored *(24)*. This type of analysis should be useful for investigation of the mechanisms of immune cell stimulation and cell-cell communication and for studying the mechanism of hormone action at the single cell level.

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Corticotropin-Releasing Activity of α-Melanotropin

Abstract. *Synthetic α-melanotropin stimulated the release of immunoreactive adrenocorticotropin from primary cultures of rat anterior pituitary cells. The effect of the α-melanotropin was dose-dependent. Cells incubated with synthetic arginine-vasopressin and α-melanotropin simultaneously produced an amount of adrenocorticotropin that was greater than the sum of the amount that the cells produced in response to each peptide added separately. Other peptides structurally similar to α-melanotropin, such as, β-, γ_1-, γ_2-, and γ_3-melanotropin, were also tested for adrenocorticotropin-releasing activity. Only the γ_3-melanotropin demonstrated a statistically significant effect. A vasopressin preparation (Pitressin, Parke-Davis) purified from posterior pituitaries and previously shown to contain some α-melanotropin was much more potent in releasing adrenocorticotropin than the synthetic vasopressin.*

Corticotropin-releasing factor was the first hypothalamic hypophysiotropic hormone to be demonstrated *(1)*, but still remains to be identified. By using primary cultures of rat anterior pituitary cells and measuring the corticotropin (ACTH) released into the incubation medium by radioimmunoassay, we observed that Pitressin (Parke-Davis), a purified vasopressin from posterior pituitaries, was much more potent than the synthetic arginine-vasopressin (AVP) in stimulating ACTH release. It was previously reported that Pitressin preparations contain α-melanotropin (α-MSH) *(2)*. Here we report the effect of synthetic α-MSH on the release of ACTH by rat pituitary cells in vitro and the interaction of α-MSH with synthetic AVP.

Our method of primary culture of rat pituitary cells is a modification of the procedure of Vale *et al.* *(3)*. The modifications can be summarized as follows. The cells are digested by a mixture of Dispase (Boehringer Mannheim, Canada), collagenase, deoxyribonuclease, and chicken serum as described previously *(4)*. The cells are then attached to polylysine-coated plastic multiwell dishes in Hepes buffered Dulbecco's modified Eagle's medium supplemented with L-glutamine and 10 percent fetal bovine serum and containing gentamycin *(5)*. The cells are then incubated for 2 days at 37°C in an atmosphere of 5 percent CO_2 and 95 percent air. The cells are washed and incubated for 2 hours before they are used. Our cell preparations contain mostly cells of the anterior lobe of the pituitary. We have found previously that the remaining cells of the intermediate lobe are more fragile than those of the anterior lobe and cannot withstand the enzymatic treatment used here to separate the cells.

For the radioimmunoassay we used an antiserum that is specific to the NH_2 terminus of ACTH and shows only weak cross-reactivity with α-MSH (0.16 percent at 50 percent displacement of ^{125}I-labeled ACTH). No cross-reactions were observed with β-, γ_1-, γ_2-, or γ_3-MSH, or with AVP. We did not identify the peptides in the incubation medium but con-

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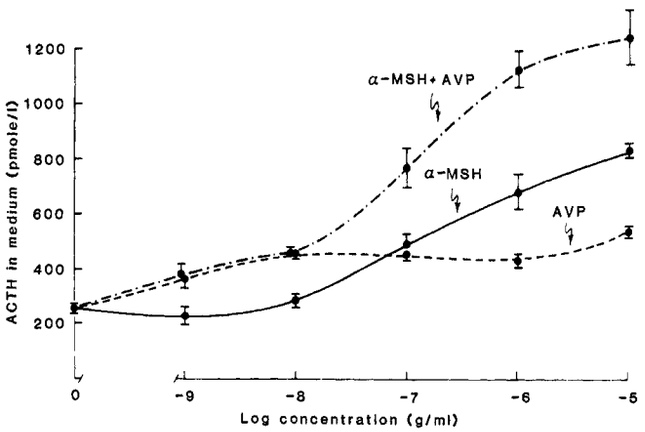

Fig. 1. Immunoreactive ACTH released into the incubation medium by 4.3×10^5 cells of the anterior pituitary from Fisher 344/Lis female rats (250 g). The data for 0.1-, 1-, and 10-μg doses were subjected to two-way analysis of variance and the function $C_{1+2} - (C_1 + C_2)$ was used as a measure of the interaction of α-MSH and AVP. The interaction was found to be significantly different from 0 ($P < .001$) by a *t*-test. The control value was calculated from 12 incubations; all the other values are the means of four incubations.

sidered all the values measured as coming from ACTH immunoreactive material. The immunoreactivity in control incubations without pituitary cells was measured simultaneously and the values corresponding to the α-MSH cross-reactivity were subtracted from the final results. α-MSH cross-reactivity did not change in the presence of the cells. The immunoreactivity of the highest α-MSH concentration used in these experiments never exceeded 20 percent of the immunoreactive material produced by the pituitary cells in culture.

In Fig. 1 we summarize our results. The effect of α-MSH was significant at doses of 0.1 to 10.0 μg/ml and was dose-dependent. The effect of synthetic AVP was significant but somewhat small compared to that of α-MSH. The effect of α-MSH and AVP together was greater than the sum of the effects of the peptides added separately. This finding led us to assay the other peptides structurally related to α-MSH (*6*, *7*). Relatively high doses (10 μg/ml) of β-MSH, γ_1-MSH, γ_2-MSH, and γ_3-MSH (*8*) were used, and the results, expressed as picograms of ACTH per milliliter of medium (mean of four determinations ± standard error), were as follows: control, 1790 ± 289; β-MSH, 2125 ± 222; γ_1-MSH, 1826 ± 257; γ_2-MSH 1997 ± 232; and γ_3-MSH 3406 ± 329. Thus only γ_3-MSH had a statistically significant effect ($P < .01$). The peptide γ_3-MSH is a part of the NH_2-terminal sequence of the precursor molecule of ACTH (pro-opiomelanocortin) (*8*) and there is some evidence that it might be released concomitantly with ACTH (*4*).

Pitressin is extracted from bovine posterior pituitaries and highly purified from oxytocin. However, the substance contains α-MSH (*2*) and is much more active than synthetic AVP (Table 1). The possibility that Pitressin contains other contaminants cannot be excluded.

The ACTH-releasing activity of synthetic α-MSH is controversial. Some investigators reported earlier that pure or synthetic α-MSH had no CRF activity (*9*). However, the presence or absence of this activity may depend on the origin and quality of the synthetic peptides, and detection of the activity may depend on the type of assay used to measure the ACTH. The reproducibility and sensitivity of our assay may be improved over earlier bioassays in vivo or assays in which pituitary halves are incubated in vitro and the ACTH measured indirectly on the basis of the amount of corticosterone produced by adrenal cortex. Other workers (*10*) have reported that α-MSH has no effect on ACTH release by cultured pituitary cells in a preparation similar to the one we used; these same workers found that β-MSH stimulated ACTH release. In our experiments β-MSH had no effect whereas α-MSH consistently increased ACTH release and potentiated the effect of vasopressin (Fig. 1). Although we think that the different mixtures of enzymes used for the dispersion of the cells might damage the receptors, other workers have reported that culturing the cells, before exposing them to the compounds to be tested, allows a certain degree of repair to take place (*10*). Nevertheless, some irreparable damage might occur as a result of the enzyme treatment and thus account for certain discrepancies in the results reported by different authors. We used α-MSH concentrations that were much higher than those found under physiological conditions.

Table 1. The amount of ACTH released by cultured anterior pituitary cells of the rat. The percentage increase of ACTH in the presence of AVP (10 μg/ml) is approximately one-half of the response obtained with Pitressin (1 U/ml). One unit of Pitressin corresponds to 2.5 μg of pure vasopressin if it is assumed that the synthetic peptide has approximately 400 U/mg. Pitressin consistently produced a much higher maximum response than pure, synthetic AVP.

Treatment	Concentration	ACTH (pg/ml)	Percentage increase*
Control	0	1584 ± 76	
Pitressin	1 U/ml	6948 ± 17	438
Control	0	1196 ± 69	
AVP	10 μg/ml	2586 ± 144	216

*With the control being considered as 100 percent.

That there may be several different corticotropin-releasing factors was suggested by Schally *et al.* (*11*). On the basis of observations with purified fractions of hypothalamic extracts it was also proposed that corticotropin-releasing activity might be an effect brought about by two different components that have little activity separately but can potentiate each other (*12*). The corticotropin-releasing activity of extracts of the rat stalk median eminence may be the result of the combination of three components, one of them being AVP (*13*); even more complex systems, including two secretion-stimulating fractions plus an inhibitor and a potentiator, have also been proposed (*14*).

We do not conclude that corticotropin-releasing factor is α-MSH, but do suggest that some fractions of purified extracts may contain α-MSH which potentiates the corticotropin-releasing activity of vasopressin. In one of the earlier observations the so-called α_2-corticotropin–releasing factor was found to be structurally almost identical to α-MSH (*15*). Our results on the ACTH-releasing activity of pure, synthetic α-MSH and γ_3-MSH lead us to believe that at least one of the components of corticotropin-releasing factor might be structurally related to α-MSH or γ_3-MSH. The significance of α-MSH having corticotropin releasing activity in vivo is difficult to predict from the present observations because of the obvious limitations of the model in vitro (*16*). However, since α-MSH appears to be produced in the hypothalamus as well as in the intermediate lobe (*17*) and since high concentrations of α-MSH can occur under certain pathological conditions, for example, in humans with ACTH-producing pituitary adenoma (*18*) and in obese (ob/ob) mice (*19*), it is possible that the corticotropin-releasing activity of α-MSH synergistic with AVP could eventually play some pathophysiological role.

Note added in proof: After this manuscript was submitted, Vale *et al.* (*20*) described the isolation and the structure of a peptide with corticotropin-releasing factor activity. Although this peptide might be the long-sought corticotropin-releasing factor, its exact physiological role remains to be established.

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6. The synthetic α-, β-, γ_1-, γ_2-, and γ_3-MSH were kindly supplied by N. Ling. Synthetic arginine-vasopressin was purchased from Peninsula Laboratories Inc. Pitressin was a gift of Parke-Davis.
7. The peptides were added in a final concentration of 10 μg/ml and incubated with 4.8×10^5 cells. The Dunnett statistical procedure was used to test the difference between control incubations and the incubations with the different MSH peptides.
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Identification of a BALB/c H-2L^d Gene by DNA-Mediated Gene Transfer

Abstract. *Gene transfer and immunoselection were used in the identification of a BALB/c genomic clone containing an H-2L^d gene (clone 27.5). Transformation of thymidine kinase–negative C3H mouse L cells with the cloned 27.5 DNA together with the herpes simplex virus tk gene produced transformants expressing L^d molecules detected by radioimmune assay with monoclonal hybridoma antibodies to L^d antigens. The foreign L^d gene products expressed by cloned mouse L cell transformants were shown to be virtually indistinguishable from BALB/c spleen L^d molecules by two-dimensional electrophoretic analysis of H-2L^d immunoprecipitates. These results indicate that the genomic clone 27.5 contains a functional BALB/c H-2L^d gene and demonstrate the usefulness of this approach for identifying the gene products encoded by cloned genes which are members of a multigene family. Furthermore, the ability to place cell-surface recognition molecules on the surfaces of foreign cells provides a powerful opportunity for functional analyses of these molecules.*

Transplantation antigens were first defined on the basis of their ability to mediate graft rejection (*1, 2*). These cell-surface molecules, which are associated with β_2-microglobulin, a 12,000-dalton polypeptide, are encoded by multiple genes of the murine major histocompatibility complex or H-2 complex (*3*). In the inbred BALB/c mouse, the H-2 complex of chromosome 17 appears to encode at least four transplantation antigens, denoted K, D, L, and R. Transplantation or class I antigens constitute one of the most polymorphic systems studied in eukaryotes. Virtually every inbred strain of mice has a distinct constellation or haplotype of class I genes. Because transplantation antigens play a fundamental role in T cell and target cell interactions (*4*), they represent an excellent model system for analyzing the genetic and molecular basis of cell-surface recognition phenomena.

Several laboratories have cloned complementary DNA (cDNA) probes for transplantation antigens (*5–8*). Class I cDNA probes were used to isolate 30 to 40 different genomic clones from a BALB/c sperm library constructed in Charon 4A lambda bacteriophage (*9*). One of these clones, designated 27.1, contained a gene that was found on restriction enzyme fragments of different sizes in differing strains of inbred mice. This restriction polymorphism was used to map this gene to the Qa-2,3 region of the Tla complex, a region that is adjacent to the H-2 complex and that encodes various hematopoietic (Qa) and T cell (TL) differentiation antigens (*9*). The DNA sequence analysis of clone 27.1, presumably a Qa-2,3 pseudogene, demonstrates that 27.1 is closely related to the classical transplantation antigens. Therefore, the 30 to 40 genomic class I clones may encode differentiation antigens in the Tla complex as well as the classical transplantation antigens of the H-2 complex. This complexity demonstrates a problem inherent in analyzing all multigene families; namely, the difficulty in correlating numerous genomic clones with their corresponding gene products, especially in view of the paucity of available amino acid sequence data.

DNA-mediated gene transfer of BALB/c class I clones into mouse L cells was used to identify the transferred BALB/c genes. Monoclonal antibodies readily distinguish the foreign BALB/c class I molecules (H-2^d haplotype) from the recipient mouse L cell transplantation antigens (H-2^k haplotype). We now report the application of this approach to the identification of a genomic clone containing the BALB/c L^d gene (clone 27.5). The nucleic acid sequence of this gene (*10*) confirms the assignment and demonstrates the usefulness of this approach in determining the coding function of cloned class I genes.

Several genomic clones were chosen for DNA-mediated gene transfer on the basis of their intense hybridization to the cDNA probes for transplantation antigens. These class I genomic clones were used independently to transform thymidine kinase–negative C3H mouse L cells (Ltk$^-$), together with the herpes simplex virus (HSV) tk gene (*11*). Transformants derived from transfections with each of these cloned genes were selected in hypoxanthine, aminopterin, thymidine

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