Inhibition of MSH Release in Frogs by Direct Application of L-Prolyl-L-leucylglycinamide to the Pituitary[1] (35805)

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Control of the release of melanocyte-stimulating hormone (MSH) in mammals (1–4), as in amphibians (5, 6), is exerted primarily by the hypothalamic inhibiting factor MIF [or MRIH (7)]. A preparation of this substance, which was purified approximately 11,000-fold from bovine hypothalamic tissue (8), was found by thin-layer chromatography to contain several compounds. Most of the biological MIF activity occurred in an area corresponding to L-prolyl-L-leucylglycinamide (PLG) (9).

A modification of the *in vivo* assay for melanocyte lightening substance (10) provided a sensitive, precise, and convenient assay for MIF. This bioassay measures the aggregation of melanin granules (lightening) in the dermal melanocytes of frogs which have been darkened by destruction of the hypothalamus (10). When large doses (10 mg) of PLG were injected into the dorsal lymph sac of the frog, or even directly into the aortic trunk (11), no lightening occurred. Marked lightening of the skin of the frog did occur, however, when smaller doses (10 ng) of PLG were applied directly to the surface of the pituitary gland. The ability of frog serum to inactivate PLG may explain some of this difference in response.

Materials and Methods. The hypothalami of adult male frogs (*Rana pipiens* collected in the North and weighing approximately 50 g) were destroyed or removed by a method previously described (10). This involved anesthesia with ether, incision of the mucosa of the dorsum of the mouth, and removal of an area of parasphenoid bone by means of a dental burr and then forceps. The incision was not closed. At least 1 day later, test material dissolved in saline was applied to the surface of the pituitary gland with a 1-μl pipette. The convenient 1-hr response approximated the maximum decrease in the melanocyte index (MI) and was used in the assay. An MI of 4.0, equivalent to 1 melatonin "unit" (10), was taken as the end point. Readings of the MI were also made at 2 or 3 hr to ensure that the test material had not permanently damaged the pituitary gland. A return of melanin dispersal in the dermal melanocytes (darkening) of the frog ruled out this possibility in each case.

Results. Injection into the dorsal lymph sac of as much as 10 mg of PLG failed to lighten frogs darkened by destruction of the hypothalamus. Since direct injection of MSH into the aortic trunk was reported to give a greater response than when the same material was injected into the dorsal lymph sac (11), this route was tried for PLG. However, 10 mg of PLG had no skin lightening effect when administered in this way. By contrast, only 10 ng of PLG reduced the MI to less than 4.0 when applied directly to the pituitary and a dose–response relationship was found. PLG did not seem to lighten isolated frog skin.

Application of 1 μg of epinephrine, 1 μg of norepinephrine, 1 μg of dopamine, 1 unit of heparin and 10 ng of melatonin (in 10% ethanol) to the pituitary gland produced melanocyte aggregation (MI <4.0). These doses were smaller than those required to produce the same degree of melanin aggregation when injected into the dorsal lymph sac (10). Histamine (10 μg), L-DOPA (10 μg), acetylcholine (10 μg), carbamylcholine (10 μg),

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TABLE I. Inactivation of L-prolyl-L-leucylglycinamide (PLG) by Incubation with Frog Serum for 10 min at 37°.

PLG groups	Melanocyte index (mean ± SEM)	p
+ fresh frog serum	4.41 ± 0.06	< 0.01
+ frog serum heated previously to 56°	3.76 ± 0.07	
Without incubation	3.66 ± 0.04	NS

serotonin (10 μg), cyclic AMP (10 μg), dibutyryl cyclic AMP (10 μg), synthetic thyrotropin releasing hormone (TRH, Abbott) (0.5 μg), lysine vasopressin (1 μg), and synthetic oxytocin (10 mU) did not produce significant lightening (MI >4.0). In every experiment, diluent was also tested; in no case did it cause lightening.

PLG was incubated for 10 min in frog serum at 37° in a Dubnoff metabolic shaker under constant gassing with 95% O_2–5% CO_2. After incubation in serum, it failed to reduce the MI below 4.0 when added at a dose which had a significant lightening effect before incubation. As a control, incubation for 10 min in saline or in Earle's solution did not reduce activity. If the serum was heated to 56° for 1 hr, cooled, and then incubated for 10 min with PLG, the skin lightening ability remained intact. Duncan's multiple range test showed no significant difference between PLG incubated for 10 min with serum previously heated to 56° and the same dose of PLG standard. The inactivation induced by incubation with fresh frog serum, however, was significant (Table I). Similar results were obtained with rat serum. The skin lightening effects of epinephrine, norepinephrine, and melatonin were not decreased after incubation with frog serum for 10 min at 37°.

Discussion. The existence of a hypothalamic inhibitor of MSH release (MIF) has been well documented (1–8). Identification of L-prolyl-L-leucylglycinamide (PLG) as the most biologically active compound in purified preparations of MIF recently was accomplished (9). This agrees with the report by Walter and his associates (12), who found that PLG had more MIF activity than other synthetic compounds tested in different assay systems.

The first isolation of PLG in purified preparations of MIF (9) was greatly facilitated by development of the assay described in this paper. The assay was capable of detecting significant biological activity in nanogram amounts of PLG, was readily reproducible, and required only 1 hr to perform. This modified *in vivo* melanocyte lightening assay, therefore, is much more convenient than those previously described (2, 13, 14).

Frogs darkened by destruction of the hypothalamus failed to lighten when large amounts of PLG were injected into the dorsal lymph sac or aortic trunk. Nanogram quantities of PLG were active in this assay, however, when applied directly to the pituitary gland. The marked difference in biological activity of PLG by these two routes of injection may be partially explained by inactivation of the material by serum. This investigation demonstrated that incubation of PLG with frog serum for 10 min resulted in significant loss of skin lightening activity. If MIF resembles other hypothalamic hormones in being released from neurosecretory nerve endings into blood vessels which penetrate the pituitary, then poor penetration of the intermediate lobe also might be involved in the decreased activity of PLG when injected systemically.

Lightening also was produced by melantonin, norepinephrine, epinephrine, and dopamine in smaller amounts when added directly to the pituitary than when injected into the dorsal lymph sac. Of these compounds, only melatonin was active in the same dose range as PLG. Among the many possible explanations, it must be considered that these substances, especially melatonin, may exert part of their melanin aggregating effect by an action on the pituitary gland. Indeed, an effect of melatonin upon pituitary MSH content has been reported, but its physiological significance is not completely clear (15). Although administration of melatonin (10) and crude preparations of MIF (15), or PLG, to darkened frogs results in skin lightening, the substances differ in several ways. Not only is the chemical structure of melatonin unlike

that of PLG, but melatonin and MIF have opposite effects upon the pituitary content of MSH as well as isolated frog skin (1–4, 8, 10, 11, 16). Furthermore, MSH affects amphibian iridiphores as well as epidermal and dermal melanocytes; whereas melatonin causes skin lightening by an effect solely upon the dermal melanocytes (17). This indicates that the action of melatonin upon frog skin cannot be completely explained by an inhibition of MSH release from the pituitary gland (16), such as would be caused by MIF.

Summary. Ten nanograms of L-prolyl-L-leucylglycinamide (PLG), when applied directly to the pituitary, resulted in lightening of a frog whose skin was previously darkened by destruction of the hypothalamus. As much as 10 mg of PLG was inactive when injected into the dorsal lymph sac or aortic trunk. Incubation of PLG for 10 min in frog serum caused significant inactivation of the material. This inactivation may partially account for the poor MIF activity observed after systemic injection of PLG. The newly modified assay described here is particularly recommended for future studies with MIF because of its simplicity.

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