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A Method for the Superfusion of Rat Hypothalami: Secretion of Luteinizing Hormone-Releasing Hormone (LH-RH)[1] (39749)

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Several workers have applied the techniques of hypothalamic incubation or of tissue culture to examine the secretion and synthesis of the releasing hormones (1-6). However, these procedures do not resolve the time course of events involved in a dynamic process such as secretion of releasing hormones; furthermore the hypothalamus is always under the influence of its own metabolic and secretory products that accumulate during the incubation.These shortcomings may be avoided by the technique of superfusion of isolated hypothalamic tissue. The development of such a procedure, following similar approaches employed for other endocrine organs, such as the adrenals (7) and the pituitary gland (8), and also for the median eminence of the newt (9), is here described. The system appears to have general applicability, but in this paper only results pertaining to the secretion of luteinizing hormone-releasing hormone (LH-RH) are discussed.

Materials and methods. Hypothalami were obtained from male rats (Sprague-Dawley strain, Laboratory Supplies Co., Indianapolis, Ind.) weighing 200 to 300 g at sacrifice and kept for at least 1 week before the experiment in an air-conditioned animal quarter with 14-hr light (on at 5:00 AM) and with adequate supplies of water and food. The animals were sacrificed by aortic bleeding, under ether anesthesia.

The medial basal hypothalamus (MBH) was dissected with fine scissors, from immediately behind the optic chiasma to the beginning of the mammillary bodies, including in depth no more than 1 mm, and kept in ice-cold superfusion medium. Usually 12 animals were sacrificed in a period of less than 30 min. At the end of the collection the MBH were split in equal halves with a razor blade. In some cases equivalent amounts of brain cortex were employed.

A schematic drawing of a superfusion chamber, made from a 5-ml plastic disposable syringe, is shown in Fig. 1. The pieces of hypothalamus are placed at the bottom of the chamber, supported by a few strands of glass wool and continuously bathed in superfusion fluid. The chamber is closed with a rubber stopper perforated by two 18-gauge steel needles (one for the inflow of air and the other for the introduction of a catheter to inject substances). The rubber stopper also allows the passage of a silicone tube (Silastic, Dow Corning Co., 0.078-in. i.d.) about 4-in. long that serves as an outlet for the fluid and air. The superfusion liquid enters the chamber from the bottom, going around and amidst the pieces of hypothalamus, and filling the barrel of the syringe with about 450 μl of medium. The air flows from a source of compressed air and forms bubbles that expel the liquid through the silicone tubing at the upper end of the chamber. The chamber is completely immersed in a temperature-regulated water bath kept at 37°.

The superfusion medium was Krebs-Ringer phosphate solution (10) to which was added 0.1% bovine serum albumin (Grand Island Biological Corp.) and 10 m*M* glucose, pH adjusted to 7.3-7.4. The pH of the collected medium in every sample of different experiments was found to remain constant over the superfusion period. A constant-speed roller pump (Holter, Extracorporeal Medical Specialties) delivered the medium at a rate of 3 ml/hr. To test the influence of calcium or potassium, the medium was modified by substituting equimolar amounts of sodium chloride for calcium

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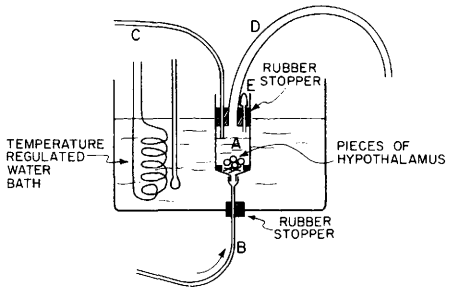

FIG. 1. Scheme of the superfusion chamber. (A) Superfusion chamber with pieces of hypothalamus on glass wool screen; (B) Tube for the inlet of superfusion medium (infused by means of a Holter roller pump); (C) Inlet for compressed air; (D) Outlet for liquid and air; (E) Needle for the passage of catheter and injection of drugs.

chloride, or by changing sodium chloride for potassium chloride.

The liquid flowing from the superfusion chamber was collected during successive 10-min periods into plastic tubes kept on ice. To each tube was added 50 μl of a 1 *N* HCl solution. The tubes were centrifuged at 4000*g* for 20 min and the supernatant was stored at −20° until the assay. The day of the assay the acid supernatant was thawed, neutralized to pH approximating 6.7 with 4.5 *N* NaOH and 0.5 *M* disodium hydrogen phosphate, and centrifuged at 4000*g* for 15 min. The amount of LH-RH was determined in duplicate, in 200-μl aliquots of the neutralized supernatant.

LH-RH was determined by radioimmunoassay utilizing a procedure similar to the one described by Nett *et al.* (11), separating the free and bound hormone by the use of ethanol, as described by Jeffcoate *et al.* (12). The antibody was kindly provided by Dr. Bernard Kerdelhué and is similar in properties to the one reported by Kerdelhué *et al.* (13). For preparation of radioactive hormone, and as a standard, synthetic LH-RH (Beckman) was utilized. The assay usually had a sensitivity of 5 pg of synthetic LH-RH. The statistical analyses of the results were carried out by the paired *t* test or the *t* test for parallel groups.

Results. The superfusion of two, four, or six medial basal hypothalami results in the release of measurable amounts of LH-RH, (Fig. 2). It is apparent that the release of LH-RH is greater at the beginning of the superfusion and decays with time until 40-60 min, when it levels and stays approximately the same for 140-180 min. The amount of LH-RH collected during the period from 60 to 120 min was used to determine a mean rate of release, that is about 0.5 pg/min for two MBH, 2.09 ± 0.28 pg/min for four MBH, and 2.07 ± 0.15 pg/min for six MBH. When two MBH are superfused many of the values fall below the level of detection of the assay, and when four MBH are employed their rate of secretion shows irregularities. Two peaks of LH-RH releases were observed in the case of four MBH. The large SE of these two pulses is due to wide individual fluctuations and the mean values are not significantly different from basal values. The preparation containing six MBH is not absolutely stable since the release changes sometimes irregularly from one collection period to another. These individual fluctuations (less than two-fold) are, however, not apparent when the average of several superfusions is calculated. It seems that the most suitable preparation for the study of experimental modifications of LH-RH release should contain six MBH. The superfusion of similar

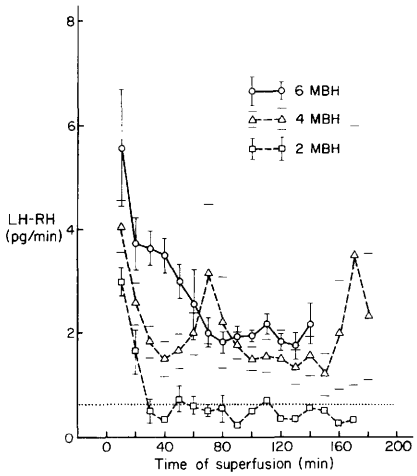

FIG. 2. Release of LH-RH from superfused rat hypothalami. Mean ± SE of the calculated rate of release of experiments performed with chambers loaded with two MBH (two experiments), four MBH (nine experiments), and six MBH (four experiments). The level of detection of the assay is given as an illustration, and it was calculated as the value of LH-RH release that gave samples with logit above 2.30.

amounts of brain cortex did not show any release of LH-RH.

There was no important change in weight of the MBH after 2.5 hr of superfusion (initial and final wet weights were 5.29 and 5.45 mg/MBH equivalent, respectively). The hypothalami contained a considerable amount of acid-extractable LH-RH at the end of the superfusion period (6.55 ± 0.44 ng of LH-RH/MBH in 10 experiments), and no differences were detected following any of the experimental manipulations described below.

To study the recovery of synthetic LH-RH in the conditions of superfusion, 500 pg were added to blank tubes or chambers containing six MBH. The blank tubes contained only superfusion medium and the LH-RH was extracted as described in Methods. In four experiments using blank tubes 60.8 ± 3.3% (mean ± SE) was recovered. In two experiments LH-RH was superfused through chambers without hypothalami and the recovery rates were 50 and 40.8%. In the case of the superfusion chambers containing MBH the LH-RH was injected at 60 or 80 min of superfusion and the recovery was calculated after subtraction of the basal release of LH-RH (assumed to be the average between the release preceding the injection and 1 hr afterwards; at that time the release of LH-RH was equal in control and LH-RH-injected chambers). In six experiments the recovery was 49.1 ± 3%. Recently, with a slightly different condition for extraction of LH-RH (neutralization to pH 4.0 approximately) the recovery from blank tubes was 100.6 ± 2.07% (4 experiments, 28 samples). With the same modification, 85.8 ± 3.0% of the LH-RH could be recovered after superfusion through chambers not containing MBH (seven experiments) and in two experiments with chambers containing six MBH the recoveries were 90.1 and 85.6%, respectively. In conclusion, there appears not to be any differences in recovery of LH-RH passed through chambers containing or not containing MBH, making it unlikely that there is destruction of secreted LH-RH. Additionally, experimental observations in our laboratory (Bledsoe and Ramirez, unpublished) indicate that enzymes destroying LH-RH are probably of intracellular localization. Summarizing, it seems that more than 80% of the LH-RH superfused from the chambers will appear at the collection tubes, from where it may be extracted with different efficiencies (100.6 or 60.8%). The results to follow were obtained under conditions of 60.8% recovery from blank tubes.

To test the superfusion system for conditions known to affect the secretion of neurosecretory cells (14), experiments were designed to examine the influence of calcium and of potassium ions. Table I presents the results of several experiments with two chambers superfused in parallel, one with normal medium and the other with calcium-free medium. It can be seen that calcium deficiency is accompanied by a progressive decline in the release of LH-RH falling to substantially nondetectable values after 40 min of superfusion. It should be noticed that the MBH were kept in normal medium up to the moment of the superfusion, thus they were exposed to calcium-free medium only once they were inside the chambers.

Figure 3 shows the results obtained by the injection into the perfusion chamber of modified medium containing a high concentration of potassium chloride (100 μl of 125 m*M* potassium that, diluted in the fluid of the chamber, should give an initial concentration of about 30 m*M* potassium). The injection into the perifusion chamber of 100 to 120 min of perifusion. It may be seen that there is a noticeable increase of release of LH-RH (over 300%) during the first 30 min; the stimulatory effect seems to have disappeared 40 min after the injection.

TABLE I. Effect of Calcium Omission on LH-RH Secretion from Superfused Chambers Containing Six MBH.[a]

Conditions	Mean rate of secretion (pg/min) for the period indicated (min)		
	0–30	40–90	100–170
Normal medium	9.9 ± 1.9[b]	3.7 ± 0.6	2.0 ± 0.5
Medium without calcium	6.9 ± 1.8	1.0 ± 0.4*	0.2 ± 0.1*

[a] Results from four parallel experiments.
[b] Mean ± SE.
* $P < 0.02$ compared with same period with normal medium.

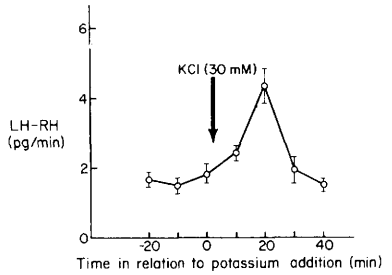

FIG. 3. Effect of increased potassium concentration on the release of LH-RH from four rat hypothalami in superfusion. The mean ± SE of five experiments for each collection period of 10 min is shown, time 0 being the moment of addition of potassium. An amount of 100 μl of modified medium containing 125 m*M* potassium chloride was injected into the chambers at 100–120 min of superfusion. There is a significant difference between the peak of potassium response and the preinjection values ($P < 0.001$, paired t test).

To further ascertain the biological validity of the preparation, the response to prostaglandin E_2, a substance that has been shown to stimulate the release of LH-RH in the living animal (15, 16) was tested. The experiments were performed running in parallel one chamber that received the injection of prostaglandin and another that received an injection of the solvent for the drug (50 μl of 5% ethanol in medium). The injection of 50 μg of prostaglandin E_2 (PGE_2) is followed by a clear and prolonged increment in the release of LH-RH (Fig. 4). If the secretions for the period from 80 to 100 min are compared between control and experimental there is a significant difference between them ($P < 0.01$). It should be noted that the duration of the response is more prolonged than the one observed under potassium stimulation. The recovery rates of LH-RH studied after PGE_2 in two experiments were of the same magnitude as the ones determined previously.

Discussion. The observation that hypothalamic tissue under *in vitro* conditions is able to release hypothalamic hormones, spontaneously and following several stimuli, has been made by several authors (1, 4, 6, 9), including one report in relation to the release of thyrotropin-releasing hormone (TRH) from the superfused hypothalamus of the newt (9). As far as we know there have not been reports of the application of the technique of superfusion to the hypothalamus of the mammal. For the male rat hypothalamus, the amount of LH-RH released under steady basal conditions was found to be small, of the order of 0.5 pg/min/MBH equivalent (or about 1.0 pg/min if allowance is made for 50% recovery). This value may be compared with a rate of release of about 7.0 pg/min/MBH equivalent in a synaptosome preparation at similar time of incubation (17). Also, from the data of Eskay *et al.* (16) we may calculate that the rate of release of LH-RH into portal vessels of the hypothalamo-hypophyseal system is about 0.4 pg/min (under pentobarbital anesthesia). From all these data it seems that the values found for the superfusion system are of the same order of magnitude as those observed in other experimental conditions and support the assumption of viable tissue.

The fact that the pieces of hypothalamus contain a high amount of LH-RH, of which only a minor portion appears to be released under superfusion (less than 3%), raises the possibility of simple diffusion as an explanation for the release of LH-RH. The results

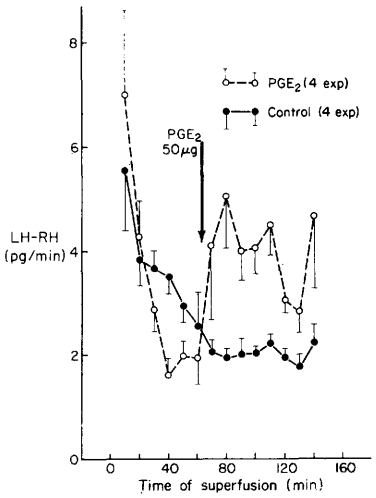

FIG. 4. Effects of prostaglandin E_2 on the release of LH-RH from superfused hypothalami of rat. The rate of release of LH-RH is shown for each collection period (10 min). The graph shows the mean ± SE for the experimental (receiving an injection of 50 μg of PGE_2 into the chamber at time indicated) and the control groups. Each group is the result of four separated experiments.

with different stimuli, however, make it very unlikely that the observed rates of release are due to diffusion or passive release of LH-RH.

The superfused hypothalamus exhibits stimulation of LH-RH release with increase in potassium ion concentration, and decrease of release under conditions of low concentration of calcium ions, indicating that it has some resemblance to the properties described for the posterior pituitary (14) and reinforcing the idea that a true process of secretion of LH-RH from living cells occurs in this system.

Further support for the proposition that the preparation contains viable neurosecretory elements comes from the experiments with prostaglandin E_2, a substance that is able to induce the secretion of LH-RH in the living animal, even if its precise site of action has not been ascertained (15, 16, 18, 19). Our results show that the isolated medial basal hypothalamus is able to respond to PGE_2, suggesting the MBH as a possible site of action in the living animal. The effect of PGE_2 appears to be quite prolonged when compared to the effect of potassium ions, but the possibility that this indicates a different mechanism of action needs the determination of a dose/response curve for PGE_2.

The system of superfusion that has been described appears very similar to the one reported by McKelvy and Grimm-Jørgensen (9) for the newt hypothalamus. They measured TRH and found clear oscillations or pulses. In contrast, we have not found significant oscillation particularly when six rat MBH were superfused. The existence of pulsatile release of LH-RH has been assumed in the castrated mammals where marked oscillation in plasma LH are found with frequency of the order of 2-4/hr (20). It would be of interest to measure LH-RH secretion rates in superfused hypothalami of castrated rats to determine whether the LH-RH released resembles the oscillatory pattern of plasma LH.

Summary. A superfusion system for hypothalamic tissue is reported and the secretion rate of LH-RH is analyzed under different experimental conditions. The responsiveness of the preparation to stimuli like high K^+, low Ca^{2+}, and prostaglandin E_2 was assessed. The usefulness of this preparation as an *in vitro* system to study the secretion rate of hypothalamic hormones is discussed.

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