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A Simple Multichannel Perifusion Apparatus of Rat Adenohypophysis and Growth Hormone and Prolactin Responses to Partially Purified Bovine Hypothalamic Extracts (39878)

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The *in vivo* (1–4) and the usual static *in vitro* method (5–10) have evidenced the existence of hypothalamic hormones which are responsible for stimulating the release of growth hormone (GH) and prolactin (PRL). However, growth hormone-releasing factor (GRF) and prolactin-releasing factor (PRF) are not yet identified. This lack of identity may be attributed to the difficulty of assaying specifically for GRF and PRF activities in hypothalamic extracts. The *in vitro* system of perifusing rat pituitary glands is superior to the static method, since it allows observation of the time-pattern response of hormones to various stimuli. In spite of this advantage, the perifusion system is not widely used because it is not always easy to operate and it is difficult to perifuse several chambers simultaneously.

In order to eliminate these difficulties, we have developed a simple multichannel perifusion apparatus for rat adenohypophysis which uses disposable syringes as chambers. This paper describes this perifusion system and the pattern of GH and PRL response to partially purified hypothalamic extracts of bovine origin which were previously suggested to possess both GRF and PRF activities (6, 10).

Materials and methods. Anterior pituitary glands were obtained from male Wistar rats weighing an average of 200 g. Macroscopically intact pituitaries were used throughout the study.

Basically, this perifusion system consists of 2 medium reservoirs, 10 sample chambers, 20 incubation chambers, 1 peristaltic pump (Manostat, Model AF-0895) with 10 cassettes (each cassette holding 2 tubings), and 1 fraction collector (Mini Collector, Mitsumi, Tokyo) specifically modified to collect 20 simultaneous samples (Fig. 1A).

As illustrated in Fig. 1B, the incubation chamber was devised from a 2.5-ml disposable syringe (i.d. 9 mm; Terumo) with the connector of a 21-gauge scalp vein set (b) (Kawasumi Kagaku Kogyo, Tokyo) attached to the tip of the barrel (a). The tubing of the scalp vein set was then cut and attached to Tygon tubing (i.d. 0.020 in.) of a sufficient length to pass through the pump cassette and finally connect with the fraction collector. Next the rubber gasket (d) of the syringe plunger (c) was pierced with a 21-gauge steel needle (f) attached to Tygon tubing (e) (i.d. 0.020 in.) which connected with the sample chamber via a three-way stopcock. Note (*): It was necessary to cut away part of the plunger base to allow insertion of the needle with attached tubing. Then, glass filter paper (h) (Whatman GF/A) was placed inside the syringe barrel near the tip. Finally the incubation chamber was completed by placing one or two pituitaries (g) on the filter paper, inserting the plunger assembly, and adjusting the volume of the medium to 0.3 ml.

The sample chamber assembly was fashioned from a 1-ml disposable syringe (Terumo) attached to a three-way stopcock as diagramed in Fig. 1C. To one of the remaining sides of the stopcock Tygon tubing (i.d. 0.025 in.) was attached. The free end of this piece of Tygon tubing was placed in the common reservoir for medium. To the second side of the stopcock,

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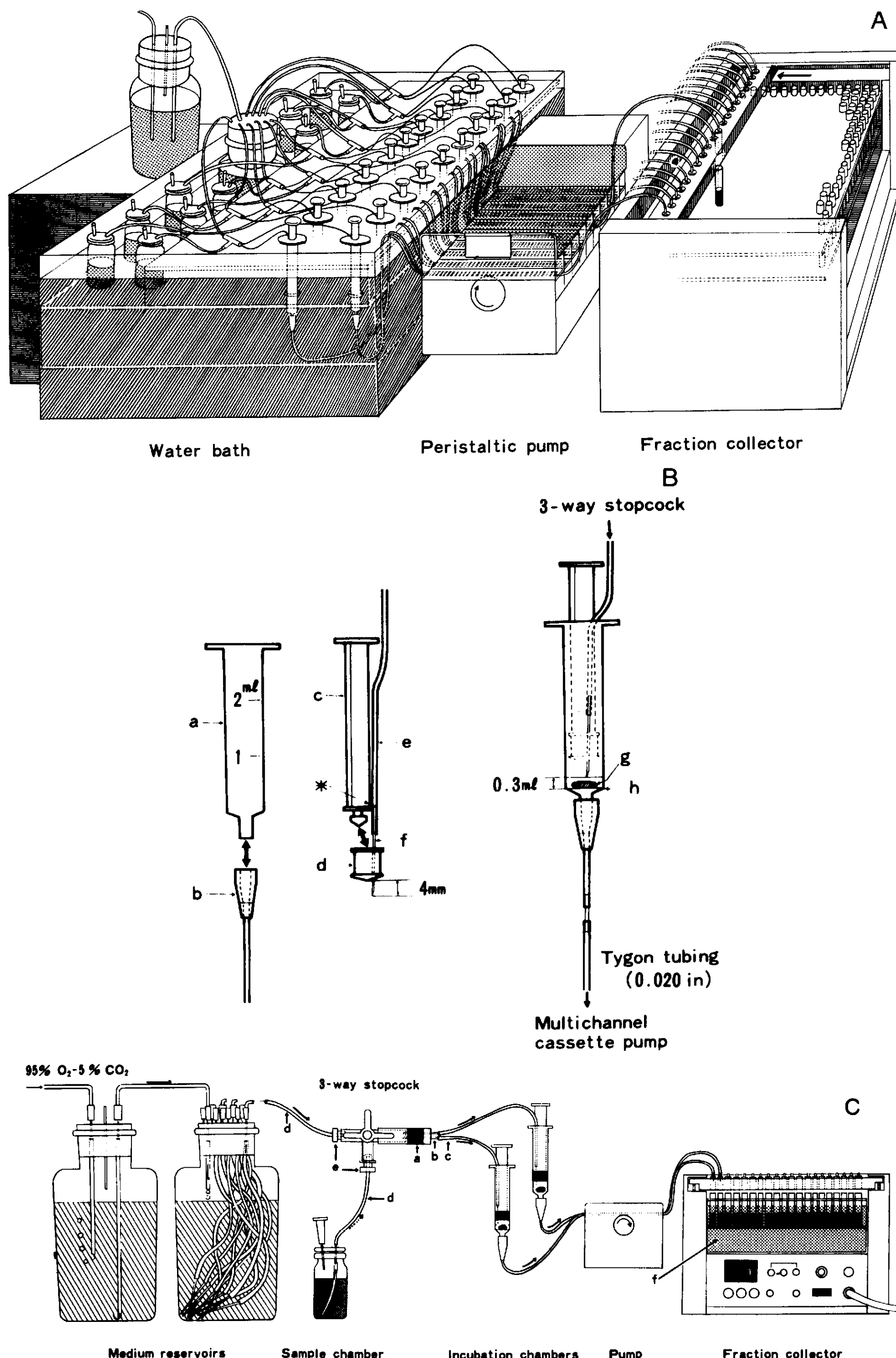

FIG. 1. (A) Multichannel perifusion apparatus. (B) Incubation chamber. The components of the unit are designated as follows: (a) barrel of 2.5-ml disposable syringe; (b) connector of 21-gauge scalp vein set; (c) plunger of 2.5-ml disposable syringe; (d) rubber gasket; (e) Tygon tubing (i.d. 0.020 in.); (f) 21-gauge steel needle; (g) anterior pituitary; (h) glass filter paper; (*) see text. (C) Diagram of the perifusion apparatus. (a) Rubber gasket taken from 1-ml disposable syringe; (b) 23-gauge steel needle; (c) Tygon tubing (i.d. 0.020 in.); (d) Tygon tubing (i.d. 0.025 in.); (e) connector (the tip of 1-ml disposable syringe barrel); (f) ice-cold water.

Tygon tubing (i.d. 0.025 in.) from a sample chamber was similarly attached. A rubber gasket taken from a 1-ml disposable syringe (a) (Terumo) was attached to the third side of the stopcock. By piercing this rubber gasket with two 23-gauge steel needles (b) it was possible to connect two incubation chambers with one sample chamber via Tygon tubing (i.d. 0.020 in.). Thus the pituitaries in 20 chambers were simultaneously perifused with this system.

As illustrated in Fig. 1A, the 20 incubation chambers, the 10 sample chambers, and 1 of the incubation medium reservoirs were all immersed in a water bath at 37°. Krebs-Ringer-bicarbonate-2 mg/ml glucose buffer (KRBG) at pH 7.4 was used as the incubation medium and was gassed with a 95% O_2–5% CO_2 mixture while in the other reservoir. The KRBG was pumped from the reservoirs through the incubation chambers by the peristaltic pump. Perifusion was carried out at a rate of 50–75 μl/min. The effluent from each incubation chamber was collected every 10 min in test tubes (i.d. 9 mm) immersed in ice-cold water on the fraction collector. All test substances were dissolved in KRBG immediately before use, equilibrated with the 95% O_2–5% CO_2 mixture, and introduced into the system by means of the three-way stopcocks. After incubation the pituitaries were weighed.

The concentration of GH and PRL in each fraction was measured by specific double-antibody radioimmunoassay using NIAMDD kits. The results were expressed as nanograms per milligram of pituitary (amount of the hormone released from 1 mg of pituitary for 10 min) in terms of NIAMD-rat GH-I-2 and rat PRL-RP-1, for GH and PRL, respectively.

Bovine hypothalamic extracts (BHE) used as test samples were fractions No. 39–42 following Sephadex G-25 gel filtration. The static *in vitro* method suggested that these BHE used had both GRF and PRF activities (6, 10).

Results. The spontaneous release of GH and PRL was stable 50 min after initiation of perifusion (Fig. 2). When the pituitary was perifused for 20 min starting at 80 min with BHE (1.0 hypothalamic equivalent) dissolved in 1.0 ml of buffer, the concentration of both GH and PRL in the effluents

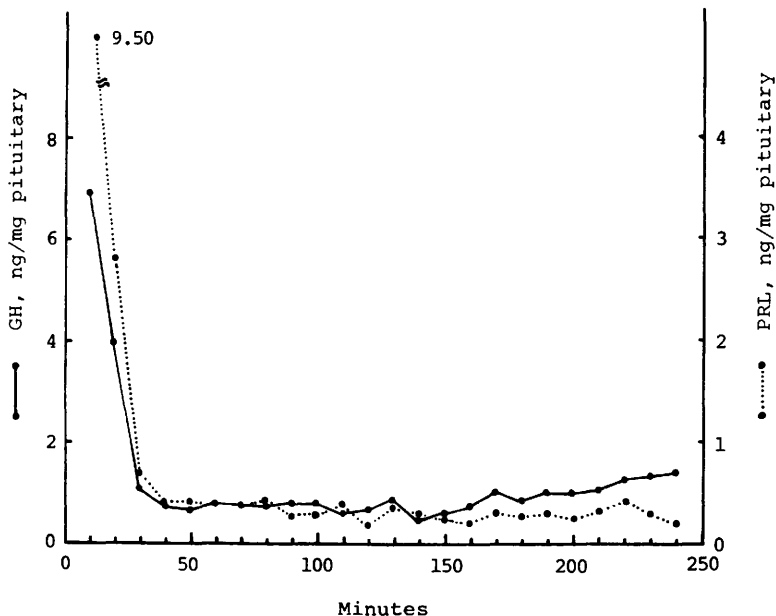

FIG. 2. Basal release of GH and PRL from perifused rat pituitary. A representative pattern of duplicate experiments, which were performed simultaneously, is shown.

increased rapidly to the highest level at the end of the perifusion, and decreased to baseline levels 30 min after withdrawal of BHE (Fig. 3A). Prolongation of the incubation interval to 40 min with the same concentration of BHE resulted in a similar elevation in the levels of both hormones until 20 min after the initial exposure to BHE. Thereafter, the levels gradually decreased, despite continuous perifusion with BHE, and returned toward baseline (Fig. 3B).

Figure 4 represents the patterns of GH and PRL responses to graded doses of BHE. BHE at a dose of 0.033 hypothalamic equivalents/ml was not effective in stimulating the release of GH and PRL. The stimulation of both GH and PRL was achieved by 0.167 hypothalamic equivalents/ml. The peak values and total amounts of both GH and PRL stimulated by BHE were dose related. When the pituitary was repeatedly perifused with graded doses of BHE in randomized order, dose-related responses of GH and PRL were observed (Fig. 5). As the administration of the same dose of BHE was repeated, both the peak value and the total amount of GH and PRL decreased (Fig. 6).

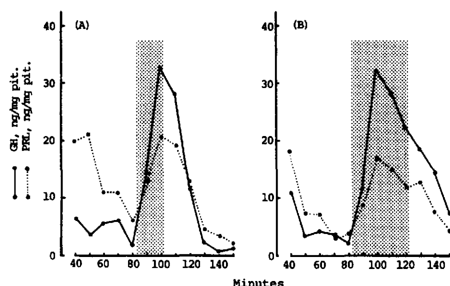

FIG. 3. The pattern of GH and PRL release stimulated by partially purified bovine hypothalamic extracts (BHE) at a concentration of 1.0 hypothalamic equivalent/ml for 20 (A) or 40 min (B). Shaded area indicates duration of infusion. A representative pattern of duplicate experiments, which were performed simultaneously, is shown. pit. = pituitary.

Discussion. The perifusion chamber, which was devised with a 2.5-ml disposable syringe, has several advantages. The chamber is economical and can be used repeatedly. It is easily built and handled. Maximally 20 incubation chambers can be operated by connecting two outlet tubings to each of the 10 cassettes on the Manostat pump. With this multiperifusion system it is possible to measure the activities of several test materials simultaneously. Thus the experimental errors which might arise from performing the same experiment on different days are greatly reduced. It may be possible that by changing the syringe size this *in vitro* perifusion system could be applied to other solitary organs such as the thyroid, adrenal glands, and ovaries.

Our previous studies using the static in-

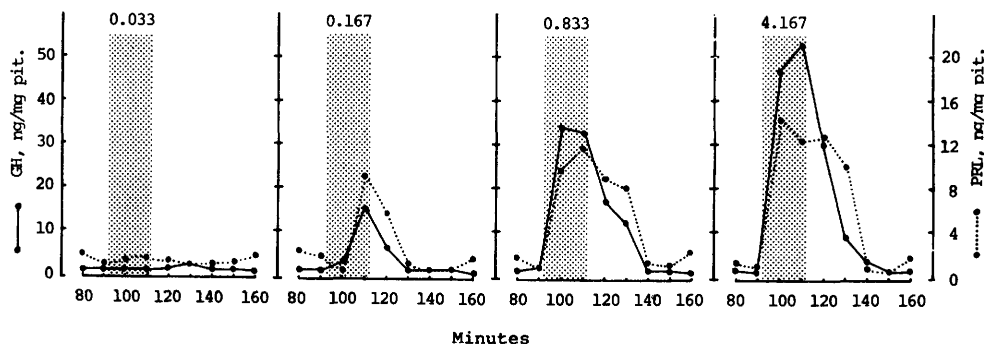

FIG. 4. The pattern of GH and PRL release stimulated by graded doses of partially purified bovine hypothalamic extracts (BHE). Shaded area indicates duration of infusion. The number above the shaded area indicates the dose of BHE in hypothalamic equivalents per milliliter. In this series of experiments eight pituitaries in eight incubation chambers, two chambers per group, were perifused simultaneously. A representative pattern of each group is shown. The total amounts of GH stimulated by 0.167, 0.833, and 4.167 hypothalamic equivalents/ml, which were calculated by adding the values above baseline level, were 22.1, 98.4, and 142.0 ng/mg of pituitary, respectively. Those of PRL were 14.5, 35.9, and 46.1 ng/mg of pituitary, respectively. pit. = pituitary.

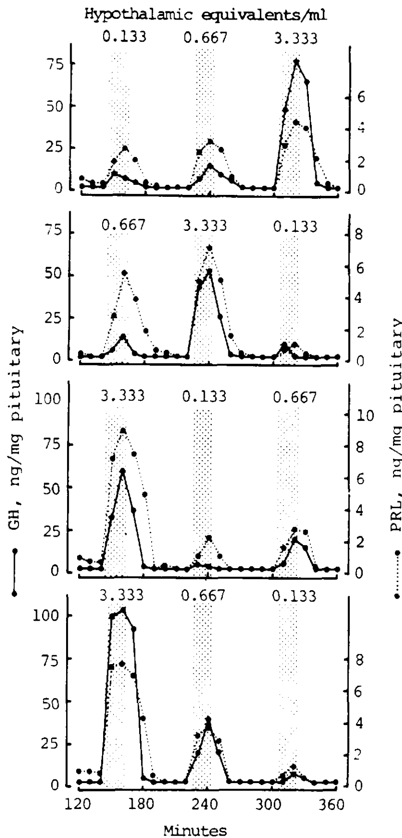

FIG. 5. The pattern of GH and PRL release stimulated by repeated pulses of partially purified bovine hypothalamic extracts (BHE). Three doses of BHE were infused in randomized order. Shaded area indicates duration of infusion. In this series of experiments eight pituitaries in eight incubation chambers, two chambers per group, were perifused simultaneously. A representative pattern of each group is shown.

cubation method (6, 10) indicated that there existed fractions containing both GRF and PRF activities in early elution following Sephadex G-25 gel filtration of bovine hypothalamic extracts. Concurring results were obtained with the perifusion system by simultaneously measuring the concentrations of GH and PRL in the effluents of these fractions of BHE. Since BHE is not highly purified it cannot be concluded that one substance in BHE is responsible for stimulating the release of both GH and PRL. However, it can be stated that the release of PRL stimulated by BHE is not due to thyrotropin-releasing hormone because the elution positions of

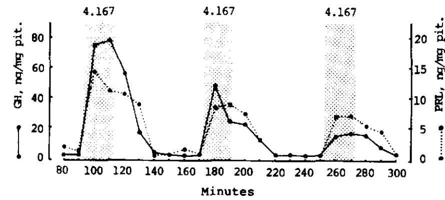

FIG. 6. The pattern of GH and PRL release stimulated by identical pulses of partially purified bovine hypothalamic extracts (BHE) at a concentration of 4.167 hypothalamic equivalents/ml. Shaded area indicates duration of infusion. The number above the shaded area indicates the dose of BHE in hypothalamic equivalents per milliliter. A representative pattern of duplicated experiments, which were performed simultaneously, is shown. The total amounts of GH stimulated by the initial, second, and third BHE were 220.8, 98.7, and 52.0 ng/mg of pituitary, respectively. Those of PRL were 41.0, 25.4, and 21.9 ng/mg of pituitary, respectively. pit. = pituitary.

BHE and thyrotropin-releasing hormone are different in Sephadex G-25 gel filtration (10). A similar observation has been made by others (3, 4, 7).

In detecting GRF activity using the *in vitro* static method, the incubation interval with BHE seemed to be very important. When the interval was prolonged from 30 min to 4 hr, the minimum effective dose of BHE was increased from 0.6 to 1.8 hypothalamic equivalents/ml (6). Therefore, it is possible that in the case of the longer incubation interval a higher amount of GH is accumulated in the medium, which reduces pituitary responsiveness to BHE. We consider the perifusion system of incubation, which immediately washes out the GH released in the chamber, to be the best way to eliminate possible influence of GH accumulated in the medium. Consequently, the total GH released was increased only four times in the static method even with 1.0 hypothalamic equivalent/ml (6). In addition, this study clearly demonstrated that the maximal stimulation by BHE was observed within 20 min after initiation of infusion, indicating that a 20-min BHE incubation was sufficient to detect GRF and PRF activities.

It is also of interest to note the response decrease which occurs with repeated stimulation by the same concentration of BHE.

There are two possibilities which might explain this phenomenon. First, a prolonged incubation interval and/or toxicity of BHE might cause cell damage. However, there is no direct evidence to indicate that BHE is toxic to the pituitary. Second, repeated pulses of BHE might cause a reduction in the readily releasable pools of GH and PRL, causing a decrease in GH and PRL release in response to prolonged or repeated administration of BHE. Further investigation is necessary to elucidate the mechanism of BHE.

Summary. A simple multichannel perifusion apparatus for rat adenohypophysis was developed using 2.5-ml disposable syringes as incubation chambers. After placing one or two pituitaries in each chamber, the pituitaries were perifused at a rate of 50–75 μl/min with KRBG which was gassed with a 95% O_2–5% CO_2 mixture.

The BHE fractions used were collected following Sephadex G-25 gel filtration of bovine hypothalamic extracts. When the pituitaries were perifused for intervals of 20 or 40 min with the BHE fractions, the peak responses of GH and PRL were always achieved within 20 min after initiation of perifusion. The peak values and the amounts of GH and PRL stimulated by BHE were dose related. Both parameters of GH and PRL decreased with repeated pulses of the same dose of BHE.

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1. Sandow, J., Arimura, A., and Schally, A. V., Endocrinology **90,** 1315 (1972).
2. Malacara, J. M., Valverde-R, C., Reichlin, S., and Bollinger, J., Endocrinology **91,** 1180 (1972).
3. Valverde-R, C., Chieffo, V., and Reichlin, S., Endocrinology **91,** 982 (1972).
4. Szabo, M., and Frohman, L. A., Endocrinology **98,** 1451 (1976).
5. Gertelendy, F., Todd, H., Peake, G. T., Machlin, L. J., Johnston, G., and Pounds, G., Endocrinology **89,** 1256 (1971).
6. Sawano, S., Matsuno, T., Yamasaki, M., Baba, Y., and Takahashi, K., *in* "Psychoneuroendocrinology" (N. Hatotani, ed.), p. 232. S. Karger AG, Basel (1974).
7. Machlin, L. J., Jacobs, L. S., Cirulis, N., Kimes, R., and Miller, R., Endocrinology **95,** 1350 (1974).
8. Nicoll, C. S., Fiorindo, R. P., Mckennee, C. T., and Parsons, J. A., *in* "Hypophysiotropic Hormones of the Hypothalamus" (J. Meites, ed.), p. 115. Williams & Wilkins, Baltimore (1970).
9. Dular, R., LaBella, F., Vivian, S., and Eddie, L., Endocrinology **94,** 563 (1974).
10. Kokubu, T., Sawano, S., Shiraki, M., Yamasaki, M., and Ishizuka, Y., Endocrinol. Japon. **22,** 213 (1975).

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