Regulation of corticotropin-releasing factor secretion in vitro by glucose

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WIDMAIER, ERIC P., PAUL M. PLOTSKY, STEVEN W. SUTTON, AND WYLIE W. VALE. *Regulation of corticotropin-releasing factor secretion in vitro by glucose.* Am. J. Physiol. 255 (Endocrinol. Metab. 18): E287–E292, 1988.—The neurosecretory responses of the isolated rat hypothalamus were assessed in vitro. Rat hypothalamic blocks were incubated for 30 min in a *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered salt solution with 5.5 mM glucose (base-line collection period). The blocks were transferred to fresh buffer with a new concentration of glucose with or without various additions (test period); corticotropin-releasing factor (CRF) and other hormones in the media were determined by radioimmunoassay. CRF secretion was maximally increased to ~200% of base line at glucose concentrations <4 mM and decreased to 65% of base line at higher glucose concentrations. The increase in CRF secretion at low glucose (0.55 or 1.38 mM) was Ca^{2+} dependent and completely reversible. Hexamethonium, cyproheptadine, and atropine partially blocked the CRF response to 0.55 mM glucose. Glucose concentrations from 0 to 11 mM had no effect on the CRF response to 47.5 mM KCl. The inhibitory effects of high glucose were completely reversed by the addition of 2-deoxy-D-glucose (3–49 mM). Glucose levels did not alter secretion of either gonadotropin-releasing hormone or arginine vasopressin from hypothalamic blocks. The results suggest that the isolated rat hypothalamus is extremely sensitive to the level of glucose and that CRF is rapidly and reversibly secreted in response to slight reductions in glucose concentrations. These concentrations are consistent with those observed during moderate to severe hypoglycemia in vivo. The rise in glucocorticoids observed in vivo during hypoglycemia may result at least in part from the ability of the hypothalamus to directly sense glucose levels and promote secretion of CRF.

rat; hypothalamus; 2-deoxy-D-glucose; vasopressin

HYPOGLYCEMIA is a potent stimulus to the secretion of adrenocorticotropic hormone (ACTH) and glucocorticoids in several mammalian species, including rats and man (2, 6, 11, 12). The maintenance of plasma glucose levels within narrow limits is arguably one of the major functions of the adrenal glucocorticoids (5). Indeed, in the dog there exists an extremely reliable inverse relationship between plasma levels of glucose and those of ACTH (12). The relative contributions of central and peripheral glucose-responsive nuclei and tissues to the generation of an ACTH secretory response to insulin-induced hypoglycemia have not been fully clarified. However, the response to peripheral hypoglycemia in the dog is greatly attenuated by infusions of glucose into the carotid arteries (13), suggesting that the region of the brain perfused by these vessels is primarily responsible for detecting the lowered level of glucose and promoting the secretion of ACTH. The hypothalamus lies within the region of the brain perfused by the carotid arteries and has long been believed to be a major site of glucose monitoring, a so-called "glucostat." Moreover, there are numerous afferent connections between hypothalamic nuclei implicated in the behavioral response (feeding) to hypoglycemia (5) and the parvocellular region of the hypothalamic paraventricular nucleus (PVN) (24). The cell bodies of this region are known to contain CRF that is ultimately transported to the media eminence (30). For this reason, we decided to examine the ability of the isolated hypothalamus to secrete CRF in vitro in response to discrete changes in the concentration of glucose in the incubation medium. In addition, the effects of glucose on the secretion of several other hypothalamic neuropeptides were also examined.

MATERIALS AND METHODS

Hypothalamic blocks (36 ± 2 mg) were removed from adult (200–250 g) male Sprague-Dawley rats (Holtzman, Madison, WI) 3–4 h after the start of the light cycle and immediately placed on ice in sterile *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Krebs salt solution, pH 7.4, containing 1 mg/ml of bovine serum albumin (BSA) and 5.5 mM glucose. The blocks extended from 1 to 2 mm anterior to the optic chiasm, to just anterior to the mammillary bodies and laterally to the hypothalamic sulci. The average depth of the blocks was 2–3 mm. Care was taken to include the median eminence. The blocks were washed two times with cold salt solution and then incubated at 37°C in 16 × 100-mm glass test tubes (3 hypothalami/tube). The incubation buffer (0.5 ml) consisted of the above salt solution with the addition of leupeptin (25 μg/ml), aprotinin (25 μg/ml), and ascorbic acid (200 μM). All incubations were performed in a humidified atmosphere of 95% O_2-5% CO_2 with gentle shaking. Under these conditions the final pH of the incubation buffer was 7.3–7.5.

The hypothalamic blocks were preincubated for 60 min with one change of buffer at 30 min. A subsequent 30-min collection (in 5.5 mM glucose) was obtained for determination of base-line CRF secretion. This value of glucose, equivalent to 0.1% (wt/vol), was chosen for the base-line reference since it is widely used in in vitro studies designed to measure hormone production and is within the high-normal range of plasma glucose in mammals. A further 30-min collection was obtained during which fresh buffer was added that contained various

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additions and one of several concentrations of glucose. In some experiments, the hypothalami were incubated in a buffer containing elevated KCl (47.5 mM) with NaCl reduced to compensate for the change in tonicity. The concentration of glucose during K^+ stimulation was maintained at whatever concentration was present in the previous collection period. In other experiments, hypothalami were incubated in either 5.5 or 1.38 mM glucose, then reincubated in the same glucose or changed from 1.38 to 5.5 mM. At the end of each incubation period, samples were immediately centrifuged to remove small pieces of tissue, acidified to pH ~ 4.5 with acetic acid and frozen in a dry ice-ethanol bath. Samples were stored at −20°C for up to 1 wk before radioimmunoassay (RIA).

RIAs. Gonadotropin-releasing hormone (GnRH) (17) and arginine vasopressin (19) were assayed as previously described. In all assays, including CRF, a volume of incubation buffer (40–150 μl) equivalent to that in the unknown was added to all standard curves. The samples were not extracted or purified prior to assay. Otherwise, CRF was determined as previously described (32). The interassay coefficient of variation for the CRF RIA was ~15%, and the sensitivity of the assay was 2–3 pg/tube. CRF released into the incubation buffer diluted in parallel with authentic rat-human CRF.

Statistical analysis. Data are expressed either as absolute values of hormone secreted per hypothalamus per 30 min or as the percent increase in secretion from a base-line collection in 5.5 mM glucose. The data are presented as percentages for the sake of clarity. However, statistical analyses were performed on the actual measured values, which conform to a normal distribution. The data from each experiment were analyzed by analysis of variance followed by Duncan's multiple range test for individual differences at the $P < 0.05$ level.

Materials. Glacial acetic acid and $MgSO_4 \cdot 7H_2O$ were obtained from Mallinckrodt (Paris, KY). Sodium bicarbonate was from J. T. Baker Chemical (Phillipsburg, NJ). Crystalline BSA was from Miles Scientific (Naperville, IL). All other reagents were from Sigma Chemical (St. Louis, MO).

RESULTS

Rat hypothalami incubated in vitro secreted CRF at a significantly elevated rate when the concentration of glucose in the medium was reduced. At glucose concentrations of 5.5 and 11.0 mM, CRF secretion was significantly less than that at 0 and 1.38 mM (Fig. 1). The concentration of glucose had no effect on the absolute amount of CRF secreted from the same hypothalami in response to a subsequent challenge with 47.5 mM KCl, although the percent increase in secretion was greater at higher glucose concentrations (Fig. 1).

Basal secretion of CRF significantly decreased with time when hypothalami were maintained in 5.5 mM glucose (Fig. 2*A*). Secretion was significantly increased when glucose was lowered from 5.5 to 1.38 mM but returned to levels not significantly different from base line after 30 min (Fig. 2*B*). When hypothalami were returned from 1.38 to 5.5 mM glucose, basal secretion was significantly reduced to levels comparable to those of hypothalami that were only incubated in 5.5 mM glucose (Fig. 2*C*).

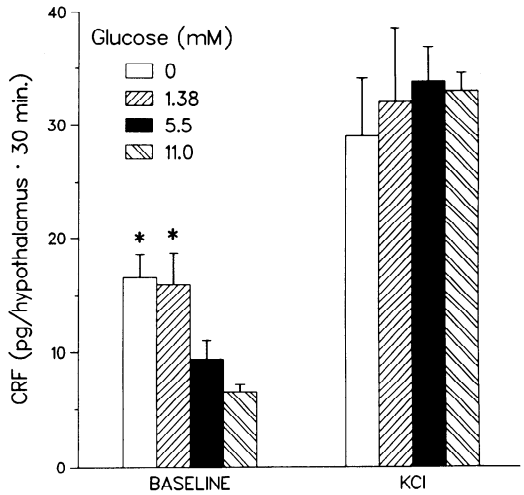

FIG. 1. Effect of glucose on basal and potassium-stimulated corticotropin-releasing factor (CRF) secretion. Rat hypothalami were incubated in HEPES-buffered Krebs salt solution with 0, 1.38, 5.5, or 11.0 mM glucose for 30 min (base line), followed by a further 30-min period with the same glucose plus 47.5 mM KCl. There was no significant effect of glucose on response to elevated KCl. Each bar is mean ± SE of 3 replicate experiments, each performed in triplicate. * Basal CRF secretion in 0 and 1.38 mM glucose was significantly higher than that in either 5.5 or 11.0 mM glucose.

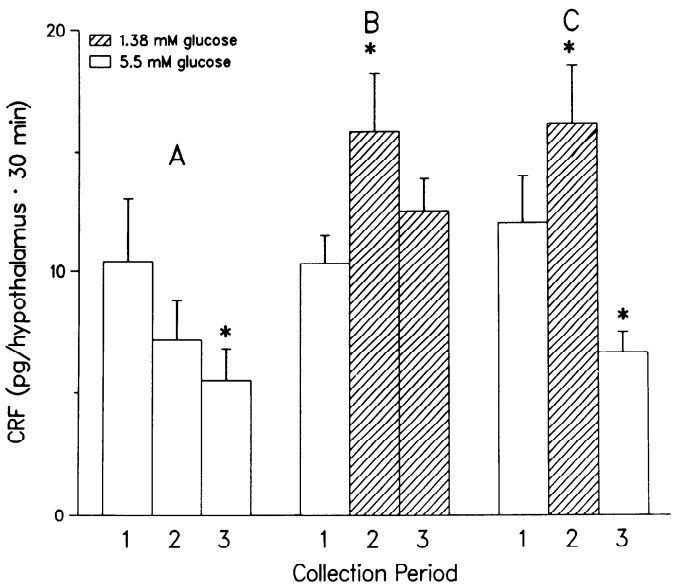

FIG. 2. Reversibility of effect of low glucose. Three groups of rat hypothalami were incubated in HEPES-buffered Krebs salt solution for 3 consecutive 30-min collection periods as detailed in MATERIALS AND METHODS. All groups were initially incubated with 5.5 mM glucose for first collection period. One group (*A*) was then maintained in 5.5 mM glucose for next 2 collection periods; the other 2 groups (*B* and *C*) were incubated with 1.38 mM glucose followed by 1.38 mM (*B*) or 5.5 mM (*C*) glucose. Each bar is mean ± SE of 3 or 4 experiments, each performed in at least duplicate. CRF, corticotropin-releasing factor. * Values significantly different from first collection period within a given group.

Maximum secretion of CRF was observed in the absence of any exogenous glucose and was 1.7 ± 0.1-fold greater than the respective base-line secretion rate in 5.5 mM glucose (Fig. 3). When glucose was changed from 5.5 mM (base line) to concentrations >4 mM, there was less secretion of CRF over 30 min compared with base

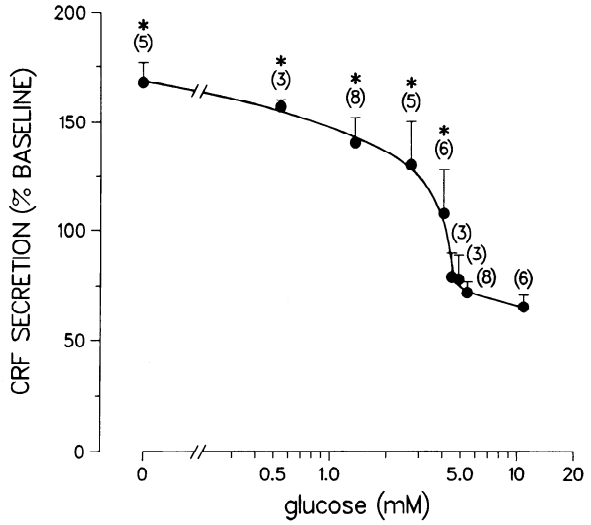

FIG. 3. Effect of different concentrations of glucose on secretion of corticotropin-releasing factor (CRF) from rat hypothalami. Three hypothalami were incubated per tube in 0.5 ml of HEPES-buffered Krebs salt solution with 5.5 mM glucose for determination of base-line CRF secretion. Subsequent collection (test period) was obtained during which glucose was changed over a concentration range of 0–11.0 mM, as detailed in MATERIALS AND METHODS. Each point is mean ± SE of 3–8 replicate experiments, as indicated by nos. in parentheses. Individual experiments were performed with at least duplicate tubes for each glucose concentration. Results are percent increase in CRF secretion during test period compared with respective base-line period (100% equals no change in secretion compared with base line). In these experiments, basal secretion of CRF was 12.3 ± 0.7 pg·hypothalamus^{-1}·30 min^{-1} (n = 47 observations from 17 experiments). * Values significantly different (at least $P < 0.05$) from the value at 11.0 mM.

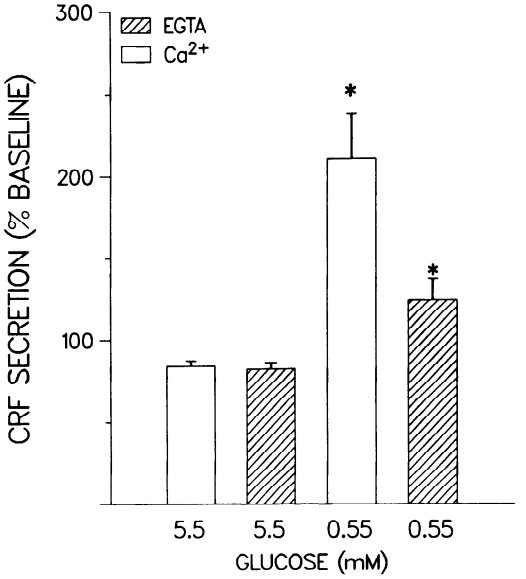

FIG. 4. Effect of calcium-free medium on corticotropin-releasing factor (CRF) secretion in low glucose. Rat hypothalami were incubated in 5.5 mM glucose to determine base-line secretion of CRF (9.3 ± 0.6 pg·hypothalamus^{-1}·30 min^{-1}). Hypothalami were then divided into 4 groups that contained either 5.5 or 0.55 mM glucose, and for each concentration of glucose, either 2.5 mM calcium or 0.5 mM EGTA. Each bar is mean ± SE of 3 experiments, each performed in triplicate. * Significantly different from all other groups.

line (<100%), with the smallest response at 11.0 mM. At concentrations of glucose <4 mM, CRF secretion was elevated compared with the respective base lines (>100%; Fig. 3). The concentration of glucose that produced no change in the rate of secretion of CRF relative to previous base line in 5.5 mM glucose, was estimated from the dose-response curve to be ~3.8 mM. When the responses at different glucose concentrations were compared with the response at 11.0 mM glucose, CRF secretion was found to be significantly greater when glucose was less than or equal to 4.13 mM (Fig. 3). Reducing the concentration of NaCl in the incubation buffer from 110.9 to 108.75 mM to match the decrease in osmolality caused by reducing glucose from 5.5 to 0.55 mM had no effect on CRF secretion (not shown).

The increase in CRF secretion that occurred when hypothalami were switched from 5.5 to 0.55 mM glucose was significantly inhibited by ~70% in Ca^{2+}-free medium (Fig. 4). However, even in the presence of ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), secretion of CRF in 0.55 mM glucose was still significantly greater than that at 5.5 mM glucose. Basal secretion in 5.5 mM glucose was not altered by removal of calcium (Fig. 4). The increase in CRF secretion produced by 47.5 mM KCl (to 377 ± 85% of base line, n = 3) was completely blocked in the absence of calcium (not shown).

The stimulatory effect of 0.55 mM glucose was inhibited by the addition of various antagonists (Fig. 5). Significant inhibition was obtained with either 10 μM or 1 mM cyproheptadine. The ganglion blocker hexamethonium also decreased the response to 0.55 mM glucose,

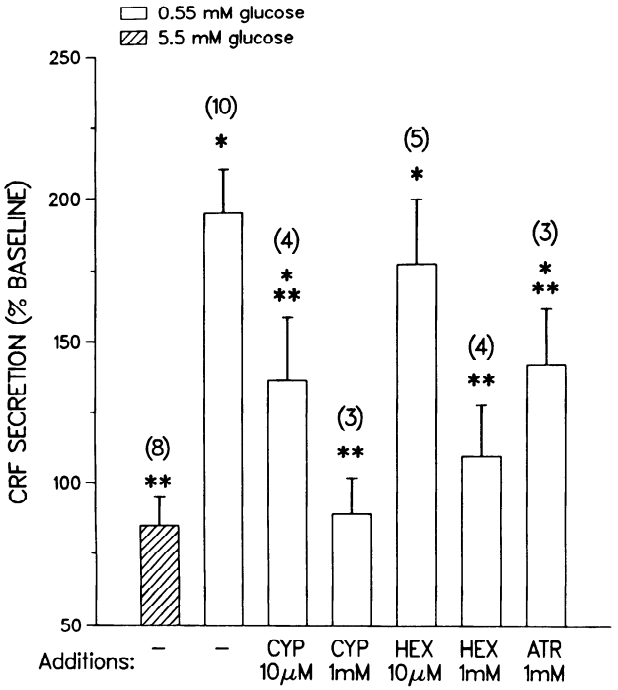

FIG. 5. Effect of neurotransmitter antagonists on the response of rat hypothalami to 0.55 mM glucose. Rat hypothalami were incubated as described in MATERIALS AND METHODS to obtain a base-line secretory rate in 5.5 mM glucose (10.8 ± 0.4 pg·hypothalamus^{-1}·30 min^{-1}). Concentration of glucose was either maintained for 30 min more at 5.5 mM (▨) or changed to 0.55 mM (□). Antagonists (HEX, hexamethonium; CYP, cyproheptadine; ATR, atropine) or vehicle were present during this final incubation period. Each bar is mean ± SE of no. of separate experiments shown in parentheses, each performed in at least duplicate. * $P < 0.05$ compared with 5.5 mM control; ** $P < 0.05$ compared with 0.55 mM control. CRF, corticotropin-releasing factor.

but the effect was only significant at 1 mM. The muscarinic antagonist atropine significantly inhibited CRF release at 1 mM; no lower concentrations were tested (Fig. 5). There was no effect of any inhibitor on CRF release that occurred in 5.5 mM glucose (not shown).

The nonmetabolizable analogue 2-deoxy-D-glucose stimulated secretion of CRF from hypothalami in vitro. When added in the presence of 11.0 mM glucose, 2-deoxy-D-glucose stimulated CRF release in a concentration-dependent manner and completely reversed the inhibition of CRF secretion caused by 11.0 mM glucose (Fig. 6).

There was no significant effect of glucose (0–11 mM) on release of gonadotropin hormone-releasing hormone from hypothalamic blocks (Table 1). Secretion of arginine vasopressin tended to be higher and more variable than that of either CRF or GnRH and although there was a trend towards reduced secretion at elevated glucose, the effect was not significant (Table 1).

TABLE 1. *Effect of glucose on secretion of GnRH and AVP in vitro*

Glucose, mM	Percent of Base Line	
	GnRH	AVP
0	71±12 (4)	140±12 (9)
0.55	ND	123±6 (7)
1.38	69±16 (4)	115±10 (10)
2.75	ND	100±11 (4)
4.13	86±7 (3)	115±25 (6)
5.55	ND	107±9 (8)
11.00	99±13 (3)	114±18 (8)

Values are means ± SE; no. in parentheses is no. of separate experiments. ND, not determined. Hypothalamic blocks were incubated in HEPES-buffered Krebs salt solution as detailed in MATERIALS AND METHODS. Medium was assayed for gonadotropin-releasing hormone (GnRH) and arginine vasopressin (AVP) by radioimmunoassay. Basal secretion (in 5.5 mM glucose) was 13.1 ± 1.2 pg·hypothalamus^{-1}·30 min^{-1} for GnRH (n = 14 observations from 4 experiments) and 97.4 ± 7.7 pg·hypothalamus^{-1}·30 min^{-1} for vasopressin (n = 52 observations from 21 experiments).

DISCUSSION

The requirement for the adrenal glucocorticoids in the maintenance of normal sugar balance in mammals is well documented. Hypoglycemia is one of the most reliable and potent activators of the brain-pituitary-adrenal axis in rats, dogs, and humans (4–7, 11–13), and at least part of the response is believed to be generated wholly within the hypothalamus (2, 13). It is at present unclear, however, which hypothalamic factor or factors are responsible for the rise in plasma ACTH that is observed in vivo after insulin-induced hypoglycemia. Experiments from this laboratory using CRF antisera have revealed that this peptide is an important component of the response to hypoglycemia in rats (19). However, in separate experiments no increase in CRF concentration was observed in portal blood of rats that were injected with insulin (19). Other investigators have observed increases in CRF in human and rat peripheral plasma after insulin-induced hypoglycemia (23, 29). These changes were attributed to CRF secreted from the hypothalamus, in part because in rats temporally coincident but opposite changes occurred in the content of CRF in the median eminence (29). However, recent studies (P. M. Plotsky, unpublished observations) indicate that peripheral plasma CRF does not originate from or reflect hypothalamic release of CRF. Recently, Smelik et al. (26) also reported a rapid drop in the content of CRF in the median eminences of rats following insulin-induced hypoglycemia. The present studies demonstrate that the isolated hypothalamus of the rat is capable of increasing secretion of CRF, but probably not vasopressin, when the concentration of glucose is decreased in the incubation medium. The apparent discrepancy between the in vitro results reported here and the previous studies on portal blood CRF cannot be fully resolved at this time but may in part be due to effects of urethan anesthesia on the secretory rate of CRF neurons that are normally responsive to changes in glucose. Alternatively, the differences may reflect the influences of afferent inputs present in the in vivo preparation but lacking in the in vitro preparation.

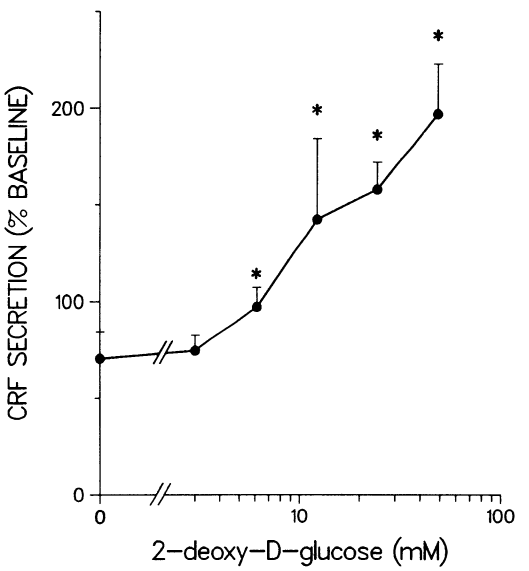

FIG. 6. Effect of 2-deoxy-D-glucose on secretion of corticotropin-releasing factor (CRF). Rat hypothalami were incubated as described in MATERIALS AND METHODS with 5.5 mM glucose to obtain a value for base-line secretion (15.0 ± 1.9 pg·hypothalamus^{-1}·30 min^{-1}). Hypothalami were then switched to 11.0 mM glucose without or with one of several different concentrations of 2-deoxy-D-glucose. Secretion of CRF during this collection period was compared with that during base-line period, and resulting percent increase is presented. Each point is mean ± SE of 3–4 experiments, each performed in duplicate. * Values significantly different ($P < 0.05$) from value obtained in absence of 2-deoxy-D-glucose.

It is unlikely that CRF secretion in response to low glucose (<4.0 mM) was the result of nonspecific events related to extreme substrate deprivation or cell death. First, hypothalami incubated in media containing 0, 1.38, 5.5, or 11.0 mM glucose all responded equally well to a subsequent challenge with 47.5 mM KCl, which would not necessarily be expected of lysed or energy-starved cells. Presumably, under these short-term conditions individual CRF neurons retain enough metabolic substrate even in the absence of glucose to permit a normal response to depolarization-induced hormone secretion. Second, the elevated secretion of CRF at 1.38 mM glucose was completely and rapidly reversed by a subsequent incubation in 5.5 mM glucose, again suggesting normal cellular integrity. Third, CRF secretion in low glucose

was attenuated by 70% in the absence of calcium. As expected from other studies (27), the effect of elevated levels of KCl was also blocked by removal of calcium. Finally, there was no effect of low glucose on GnRH (or vasopressin) secretion, confirming earlier studies on GnRH by Lengyel et al. (14) and suggesting that the effect was not the result of generalized cellular damage or death. In addition to these considerations, it is worth noting that the slight change (1.5%) in osmolality caused by reducing glucose from 5.5 to 0.55 mM cannot account for the changes observed in secretion of CRF, since reducing sodium in the buffer to match this change in osmolarity had no effect on CRF release.

The inhibitory effect of glucose was concentration dependent, with half-maximal and maximal inhibition at ~4.0 and 11.0 mM, respectively. High glucose levels apparently do not inhibit CRF release by an action that leads to the death or severe damage of neurosecretory cells, since the effect of a maximally inhibitory concentration of glucose (11.0 mM) can be completely reversed with the nonmetabolizable analog 2-deoxy-D-glucose, which competitively antagonizes both the entry of glucose into cells and isomerization of glucose 6-phosphate. The present studies do not uncover the mechanism of the inhibitory action of glucose on CRF secretion. In pancreatic A-cells, glucose also acts as an inhibitor by reducing secretion of glucagon (18). This action may be associated with a decrease in both basal and stimulated levels of intracellular Ca^{2+} (10). In view of the calcium dependence of CRF release at low glucose observed in the present studies, it is possible that high levels of glucose may act within the hypothalamus in a manner analogous to the action of the sugar in islet A-cells.

It is noteworthy that at 4 mM (75 mg/dl) glucose there was a nearly stable secretory rate of CRF (no increase or decrease compared with base-line levels). This concentration is well within the normal range of basal levels of glucose observed in most mammals, including the rat. The slope of the response curve is steepest in this region, suggesting an exquisite sensitivity of the hypothalamus to changes in glucose around normal physiological levels. The magnitude of the CRF response to low glucose, although small (approximately twofold), is similar to the response observed in portal blood in rats subjected to a severe hemorrhage stress (21). In the latter case, a twofold rise in portal blood levels of CRF leads to a dramatic increase in plasma ACTH, even in the presence of antagonists to arginine vasopressin (21). However, our data do not exclude the possibility that other factors besides CRF may contribute to the ACTH response to hypoglycemia in vivo. Indeed, a twofold increase in CRF in vitro represents a small portion of the total dose-response curve for ACTH secretion from cultured rat pituitary cells (31). The effect of CRF on release of ACTH is known to be potentiated by various neurotransmitters and peptides (3, 8, 9). The most studied of these, arginine vasopressin, is known to increase in peripheral and portal plasma in vivo after insulin-induced hypoglycemia in dogs (12, 13) and rats (4, 19). The failure to observe a significant increase in vasopressin secretion at low glucose concentrations in the present studies may in part be attributed to the fact that in vivo, the rise in vasopressin after insulin injection (in dogs) appears to result not from the decreased plasma glucose levels per se but rather to insulin-induced changes in blood volume and plasma sodium (4, 13). In this respect, however, it is important to note that raising or inhibiting endogenous vasopressin levels in man (by varying plasma osmolality) has no effect on the subsequent ACTH and cortisol responses to insulin-induced hypoglycemia (1), suggesting that vasopressin secreted from the neural lobe of the pituitary is not an important component of the ACTH response to hypoglycemia in man. However, it is unknown at present whether the source of vasopressin that is secreted directly into portal blood is also under osmotic control. Nevertheless, it is clear from our studies that the isolated hypothalamus of the rat contains all the components needed to rapidly detect changes in glucose levels and to subsequently promote secretion of CRF.

The intrahypothalamic site of the glucose sensor has long been believed to be located within the ventromedial nuclei (VMN). The present studies do not distinguish definitively between a direct effect of glucose on CRF-containing cell bodies and an indirect effect via the VMN or some other hypothalamic region. The observation that the CRF response to 0.55 mM glucose could be partially or totally blocked by various antagonists, although at high concentrations, suggests that low glucose levels activate interneurons capable of promoting secretion of CRF. The requirement for high concentrations of antagonists probably reflects the use of intact tissue, rather than dissociated cells, which would be expected to show greater sensitivity. More than one interneuron may be involved in the response to low glucose, since the response was at least partially inhibited by both cholinergic and serotonergic antagonists. The effect of cyproheptadine is somewhat puzzling, since to our knowledge there are no serotonergic perikarya located within the hypothalamus. However, a well-documented serotonergic terminal field has been identified at the level of the PVN, with fibers originating in the B7–B9 raphe nuclei (25). It is conceivable, therefore, that removal of the hypothalamus from the brain may lead to leakage of serotonin from terminals of severed axons and that tonic levels of this transmitter are necessary for the full response to low glucose. Alternatively, the terminals themselves may remain functionally intact and capable of responding to changes in glucose, perhaps indirectly through presynaptic receptors. Both acetylcholine (or its nonhydrolyzable analogues) and serotonin have been shown by several investigators to increase CRF secretion both in vitro and in vivo (15, 16, 20, 28), and the cholinergic response appears to be mediated via both muscarinic and nicotinic receptors (15, 28).

It is proposed, therefore, that the rat hypothalamus, isolated from all inputs from both peripheral and central (extrahypothalamic) glucose sensors, is capable of responding rapidly and reversibly to discrete changes in glucose levels with secretion of CRF but probably not of vasopressin. The stimulation of CRF secretion at low glucose appears to be mediated indirectly through intrahypothalamic cholinergic and serotonergic inputs. It is interesting to speculate that the inhibition of CRF release by glucose may represent a final link in a classical

feedback loop; hypoglycemia stimulates secretion of CRF, ACTH and glucocorticoids, which leads to an increase in plasma levels of glucose, which in turn acts to decrease further CRF release. In an analogous situation, hypotension is a powerful stimulus to ACTH release, and hypertension can partially inhibit the ACTH response to a different stressor (22).

The authors thank Carolyn Douglas for expertise in performing the CRF radioimmunoassay, Gayle Yamamoto and Anne Corrigan for supervising the collection of adult rat hypothalami, and Susan McCall for assistance with the manuscript.

This work was supported by National Institutes of Health Grants DK-26741, DK-07563, and DK-33093. Research was conducted in part by the Clayton Foundation for Research, California Division.

P. M. Plotsky and W. W. Vale are Clayton Foundation Investigators.

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Received 16 November 1987; accepted in final form 5 April 1988.

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