Hyposmolar Stimulation of Secretion of Thyrotropin, Prolactin, and Luteinizing Hormone Does not Require Extracellular Calcium and Is not Inhibited by Colchicine, Cytochalasin B, Ouabain, or Tetrodotoxin

(43026)

MONTE A. GREER, SUSAN E. GREER, AND SAKAE MARUTA
Section of Endocrinology, Department of Medicine, Oregon Health Sciences University, Portland, Oregon 97201

Abstract. **Hyposmolar stimulation of thyroid-stimulating hormone, prolactin, and luteinizing hormone secretion by dispersed perifused rat pituitary cells was not depressed by removal of Ca^{2+} from the perifusion medium or by 0.1 m*M* colchicine, 20 μM cytochalasin B, 0.1 m*M* ouabain, or 3 μM tetrodotoxin. The secretory response induced by medium hyposmolarity or by thyrotropin-releasing hormone was not appreciably different at 23, 37, or 43°C, but was markedly reduced or abolished when the experiments were performed at 1°C. These data indicate that microtubules or microfilaments, transport of extracellular Ca^{2+} into the cytoplasm, and plasmalemma ion transport mechanisms sensitive to ouabain or tetrodotoxin are not essential components of the mechanism by which extracellular hyposmolarity induces secretion.** [P.S.E.B.M. 1990, Vol 193]

In studies of the dynamics of *in vitro* hormone secretion of dispersed rat adenohypophyseal cells, we have found that diluting the medium with as little as 2% distilled water stimulates secretion of all hormones we have measured (growth hormone, prolactin, thyroid-stimulating hormone, luteinizing hormone, adrenocorticotropic hormone) with an immediate secretory response indistinguishable from that induced by specific hypothalamic secretagogue hormones (1). Secretion is proportional to the degree of hyposmolarity and is not due to dilution of the medium ingredients since no secretion is induced if isosmolarity is maintained by dilution with 5% aqueous mannitol. Hyperosmolarity sometimes suppresses basal secretion of hormone, but return to isosmolarity consistently results in a secretory burst indistinguishable from that induced by hyposmolarity and which is proportional to the degree of preceding hyperosmolarity (2). The mechanism of hyposmolar induction of secretion is unknown and could be due to a variety of factors. In this study we have investigated the role of extracellular calcium and the effect of agents which alter ionic flux and cytoskeletal integrity. Some of these data have previously been reported in preliminary form (3, 4).

Received April 3, 1989. [P.S.E.B.M. 1990, Vol 193]
Accepted November 16, 1989.

0037-9727/90/1933-0203$2.00/0

Materials and Methods

Perifusion of acutely dispersed anterior pituitary cells from 2-month-old female Simonsen Sprague-Dawley rats in unselected stages of the estrous cycle was performed in a 0.2-ml column as described previously (5). An initial equilibration perifusion of Dulbecco's modified Eagle's medium containing 10% horse serum (DMEM; Gibco) alone or containing the drugs tested as appropriate was performed for 45 min before the start of each experiment. All perifusion media were continuously gassed with 95% O_2-5% CO_2 beginning 1 hr before the experiment. Flow rate was 0.5–1 ml/min. Fractions were collected at 1-min intervals. The media reservoirs and perifusion chamber were maintained at 37°C in a water bath. The DMEM was made hypotonic

by diluting with the same glass-distilled deionized water used to constitute the DMEM. When drugs (e.g., colchicine) were dissolved in the medium, the water used to dilute the medium contained the same concentration of drug to keep this variable constant. In some experiments, mannitol was dissolved in the medium to produce hyperosmolarity. Osmolarity of the solutions was determined by freezing point depression with an Advanced Instruments Model 3W Wide Range Osmometer with a sensitivity and accuracy of 1 mOsm. The osmolarity of DMEM was 325 mOsm. Addition of distilled water produced a closely proportional reduction in osmolarity (e.g., 50% water added to DMEM produced a solution of 163 mOsm, addition of 25% water made a solution of 236 mOsm). Addition of mannitol to DMEM produced: 0.5% = 349 mOsm; 1% = 379 mOsm; 2% = 433 mOsm; 4% = 547 mOsm. Addition of 2% mannitol or 30% water was thus approximately equivalent in changing medium osmolarity in opposite directions. Toxicity of the hyposmolar solutions was checked by measuring trypan blue staining of the cells after incubation in a 30% hyposmolar DMEM solution for various times. Compared with cells incubated in isosmolar DMEM for the same time, there was no cell death after incubation in hyposmolar medium for 10 min and only 15% cell death for hyposmolar incubation for 1 hr, much longer than we employ in any of our experiments.

Cytochalasin B, ouabain, and colchicine were obtained from Sigma, tetrodotoxin from CalBiochem. Thyroid-stimulating hormone (TSH), prolactin (PRL), and luteinizing hormone were measured with rat radioimmunoassay kits supplied by the National Hormone and Pituitary Program, NIDDK. The stated potency of the standards supplied was used in the calculations. All samples from each individual experiment were analyzed in the same single-point assay to avoid interassay variance. Intraassay coefficient of variance was <10% in all assays. In most experiments both TSH and PRL were measured in all fractions. In some experiments luteinizing hormone was measured. There was no significant difference between the hormones in the secretory pattern induced by osmolar changes. In experiments measuring the integrated response to various concentrations of agonist, the hormone secreted during the 4 min of basal secretion immediately preceding perifusion of the agonist were subtracted from the stimulated secretion during the first 4 min of exposure to agonist to give the net secretion due to agonist. All experiments were performed at least twice with essentially identical results each time except for the experiment with tetrodotoxin, which was performed only once.

Results

Replicability and Refractory Period of Secretory Response to Hyposmolarity. A 1-min exposure to a 20% reduction in medium osmolarity was repeated four consecutive times at progressive intervals of 5, 10, 15, and 20 min (Fig. 1). Essentially the same secretory response was induced with each exhibition of the hyposmolar stimulus, although there was insufficient time for the secretory rate to return to baseline with 5-min intervals. We have previously shown that continuous exposure to a given concentration of thyrotropin-releasing hormone (TRH) or degree of hyposmolarity causes an immediate high amplitude secretory burst followed by a low level of sustained secretion (5). The present data indicate the refractory period for a high-amplitude secretory response to hyposmolar stimulation is <5 min, similar to that for TRH (5). There was an approximately 50% decrease in basal secretion of PRL during the perifusion period, as is usually seen in our laboratory with freshly dispersed cells which have an initial high PRL secretion rate, presumably because of the acute loss of hormones which inhibit PRL secretion. The absolute secretory amplitude in response to the hyposmolar stimulus remained constant, but the decrease in basal secretion with time magnified the relative height of the secretory response.

Effect of Removal of Ca^{2+} from the Medium. Because of the expense involved, we were unable to obtain Ca^{2+}-free DMEM. However, when DMEM containing 3 or 60 m*M* EDTA, sufficient to chelate several-fold more Ca^{2+} than contained in the DMEM, was perifused there was no inhibition of the luteinizing hormone (Fig. 2), PRL, or TSH responses to hyposmolarity but the PRL and TSH secretory responses to TRH were markedly reduced. In other experiments using a simpler medium of Krebs-Ringer-bicarbonate, if the Krebs-Ringer-bicarbonate was made without Ca^{2+} similar results were obtained as when using DMEM plus EDTA.

In repeated experiments comparing the effect of TRH and hyposmolarity, there was a highly significant difference between the requirements for extracellular Ca^{2+} of these two agonists (Table I). The removal of

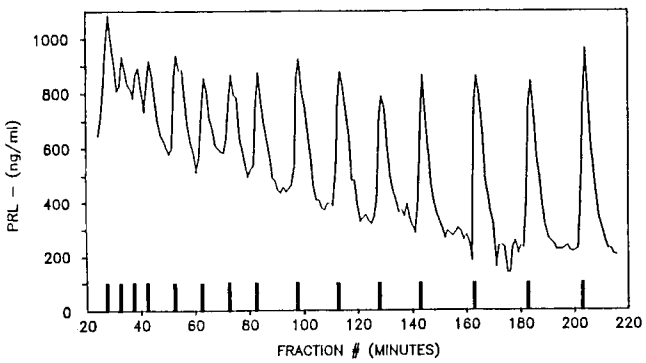

Figure 1. Replicability and refractory period of PRL secretory response of perifused pituitary cells to hyposmolarity. Each vertical bar indicates a 1-min perifusion of DMEM diluted 20% with water.

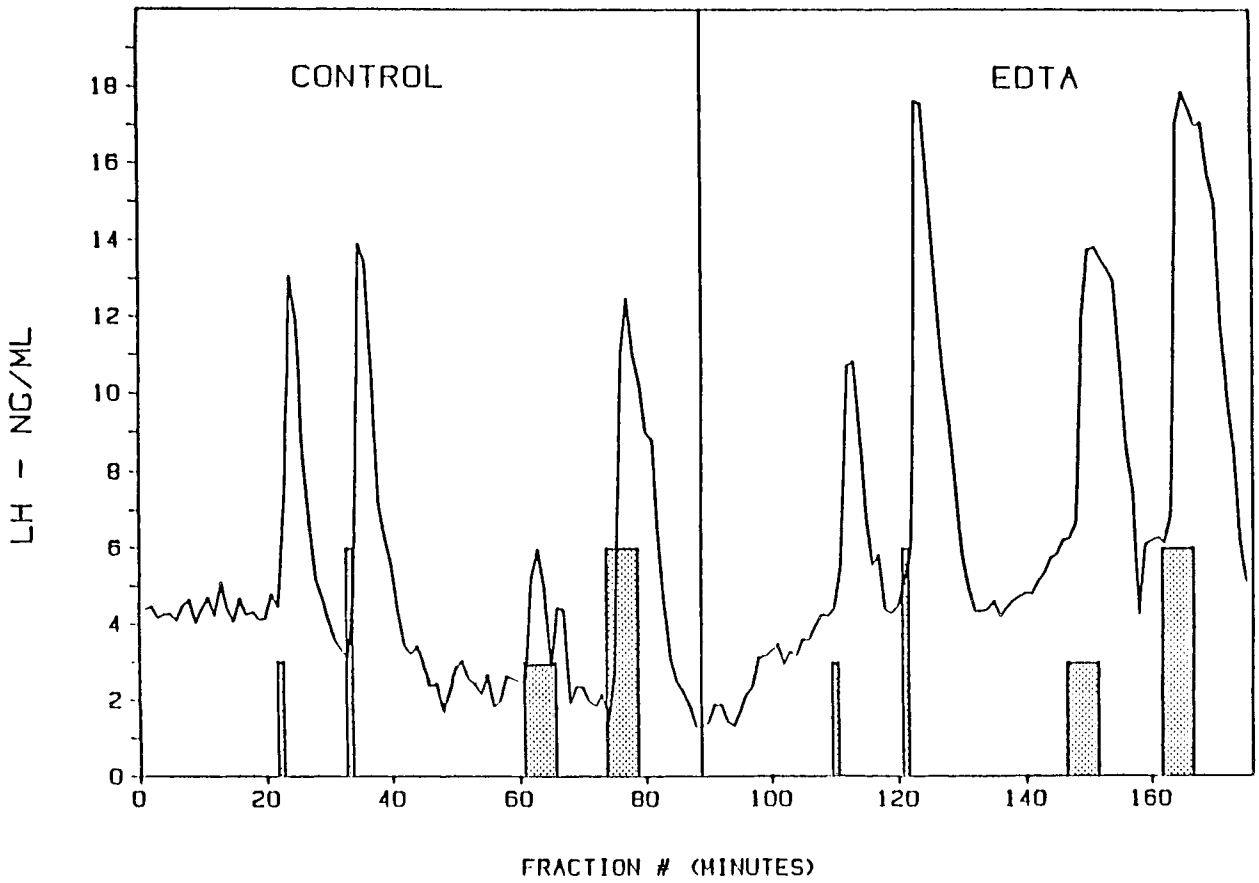

Figure 2. Acutely dispersed adenohypophyseal cells were perifused for 1 or 5 min with DMEM diluted with 10 or 20% water, as indicated by the width and height, respectively, of the stippled bars. At the vertical line separating the panels, the perifusing medium was changed to DMEM containing 3 m*M* EDTA.

Table I. Effect of $[Ca^{2+}]_e$ on Nanograms of Hormone Secreted in Response to Hyposmolarity or TRH[a]

	TSH			PRL		
	n	Ca^{2+}		*n*	Ca^{2+}	
		+	−		+	−
Hyposmolarity (%)						
5	2	1.7	1.7	3	0.0	8.0 ± 6.3
10	3	2.2 ± 1.7	3.1 ± 2.0	4	3.2 ± 2.4	13.3 ± 8.9
20	3	4.4 ± 2.9	6.6 ± 3.6	4	11.0 ± 5.8	21.2 ± 14.8
30	1	1.2	3.8	1	3.8	10.6
		$P = 0.012$			$P = 0.009$	
TRH (n*M*)						
0.1	2	0.5	0.1	4	1.2 ± 1.1	1.1 ± 1.0
1	2	3.4	0.1	4	5.8 ± 4.1	0.2 ± 0.2
10	2	4.5	0.1	4	15.6 ± 4.9	0.3 ± 0.2
100	1	5.7	0.2	2	36.8	8.4
		$P < 0.001$			$P = 0.007$	

[a] Data (mean ± SE) are combined from the number of experiments indicated under *n*. Variance is high because the experiments were performed at different times. The data are paired from experiments in which the same pool of cells was perifused with medium with a normal Ca^{2+} concentration (+) or without Ca^{2+} plus 0.1 m*M* EDTA (−). The probability value in each group indicates Student's paired *t* comparison of the effect of Ca^{2+} for the combined pool containing each stimulus intensity within that group.

extracellular Ca^{2+} not only did not reduce the stimulation of TSH and PRL secretion by hyposmolarity but actually produced a significant increase in this effect. This contrasts with the marked decrease in TRH-induced secretion produced by reducing extracellular Ca^{2+} (Table I).

Colchicine and Cytochalasin B. Addition of 0.1 m*M* colchicine or 20 μM cytochalasin B did not alter the secretory response to hyposmolar stimulation (Figs. 3 and 4), indicating that perturbation of the microtubule or microfilament system of the dispersed cells does not affect the secretory response to hyposmolarity.

Ouabain and Tetrodotoxin. Addition of 0.1 m*M* ouabain or 3 μM tetrodotoxin to the medium also did not alter the secretory response to hyposmolarity (Figs. 5 and 6), indicating that the hyposmolar stimulus is not dependent on the transplasmalemma sodium flux affected by these substances.

Combined Exposure to TRH and Hyper- or Hyposmolarity. Various combinations of TRH with osmolar changes are shown in a representative study in Figure 7. During continuous perifusion of TRH, both hyposmolarity and hyperosmolarity induced essentially the same net secretory response as if they were given alone. Similarly, TRH produced the same net increase in secretion when given alone or in the presence of a hyper- or hyposmolar perifusate. This could indicate that hyposmolarity and TRH have different mechanisms of action which are additive. However, a clear interpretation is not possible since addition of a higher concentration of TRH to a continuous perifusion of a constant TRH concentration (5) or of a greater degree of hyposmolarity to a continuous perifusion of a constant degree of hyposmolarity (unpublished data) will produce an additional high-amplitude secretory burst as a "staircase" phenomenon indicating "all or none" responses of multiple secretory units with different response thresholds (5, 6). The additive effects seen in this experiment with different types of agonist thus could also indicate that TRH and hyposmolarity have a similar mechanism of action.

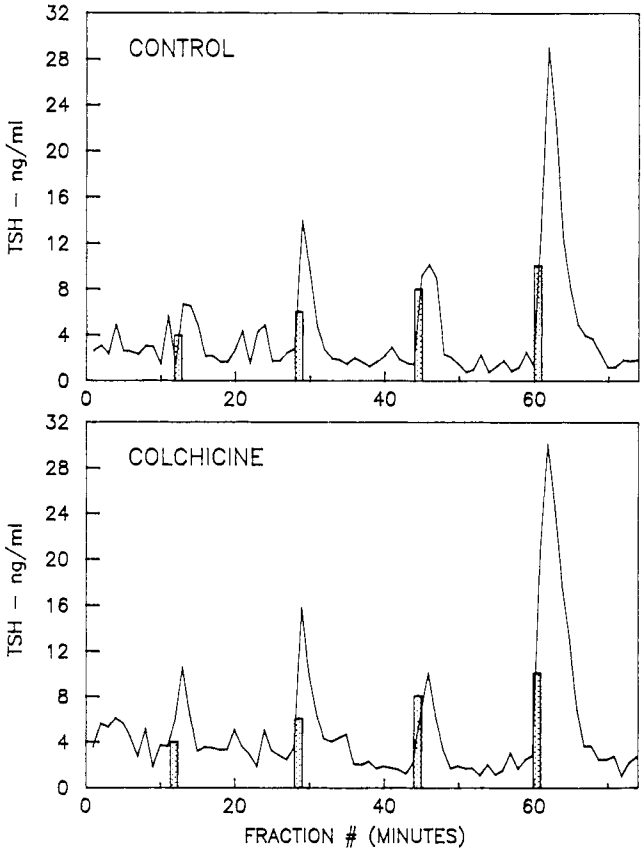

Figure 3. Parallel pituitary cell columns were perifused with DMEM without (upper panel) or containing (lower panel) 0.1 m*M* colchicine. One-minute perifusions of medium diluted with 4, 8, 16, or 32% water were given as indicated by the relative height of the stippled bars.

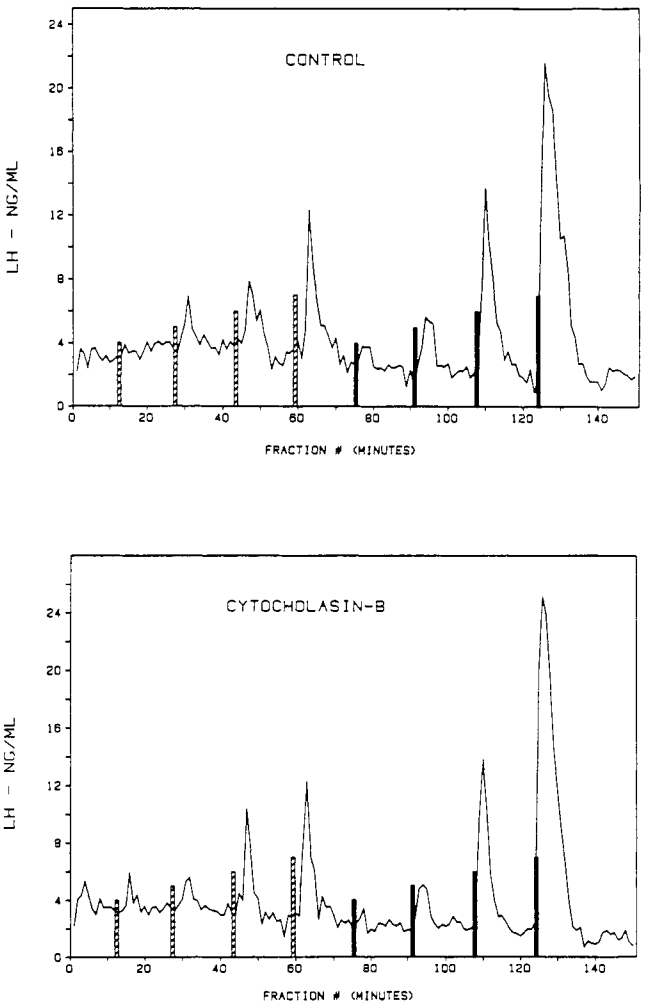

Figure 4. Acutely dispersed adenohypophyseal cells were perifused with DMEM alone (upper panel) or DMEM containing 20 μM cytochalasin B (lower panel). Medium to which 0.25, 0.5, 1, or 2% mannitol (hatched bars) or 4, 8, 16, or 32% water had been added (solid bars) was perifused for 1 min each, as indicated by the spacing and height of the vertical bars. Hyperosmolarity from addition of mannitol produced a typical "off-response" (2).

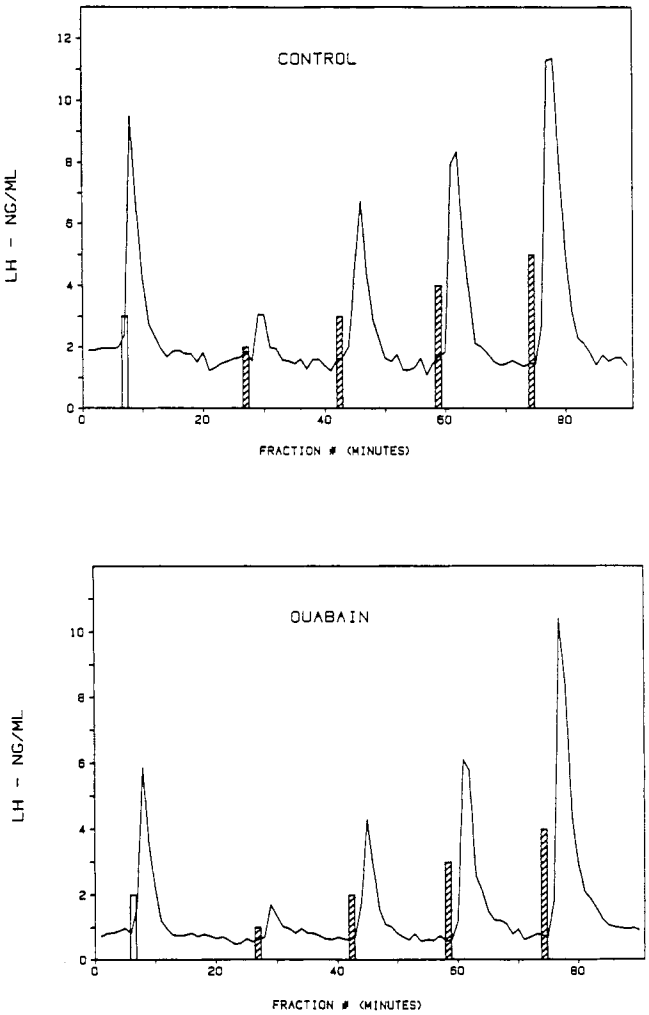

Figure 5. Parallel pituitary cell columns were perifused with DMEM without (upper panel) or containing (lower panel) 0.1 m*M* ouabain. Medium diluted with 10% water (open bar) or containing 0.5, 1, 2, or 4% mannitol (hatched bars) was perifused for 1 min each.

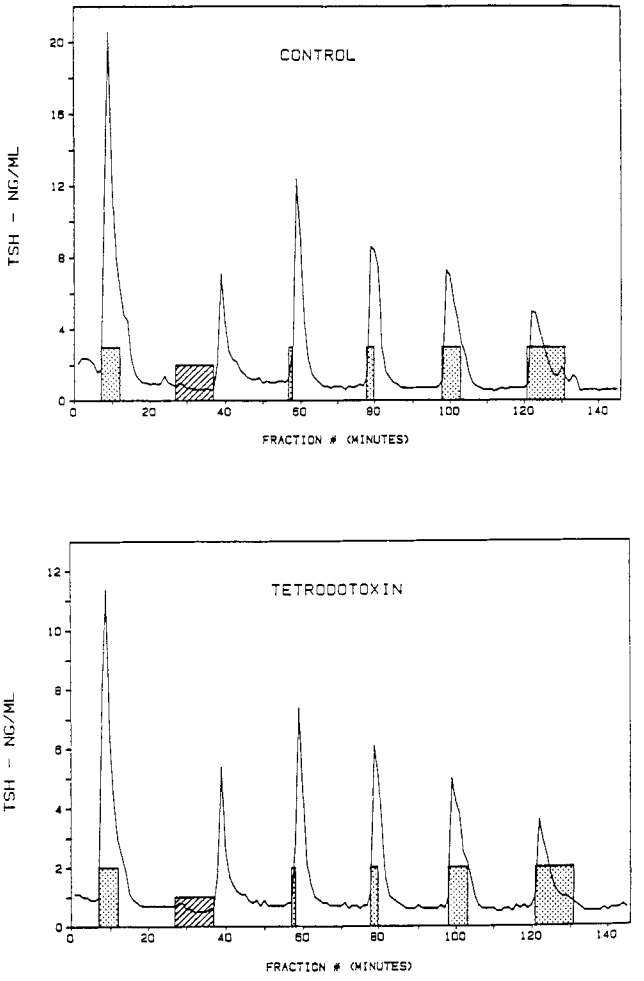

Figure 6. Parallel pituitary cell columns were perifused with DMEM without (upper panel) or containing (lower panel) 3 μM tetrodotoxin. Medium containing 2% mannitol (hatched bar) or diluted with 20% water (stippled bars) was perifused for 1, 2, 5, or 10 min, as indicated by the width of the bars.

Temperature Dependency. Both hyposmolarity and TRH stimulated secretion to approximately the same degree at 23, 37, and 43°C. However, stimulation was markedly reduced at 1°C; the response to osmolar changes was undetectable and that to TRH was almost completely abolished.

Discussion

We have previously suggested that external hyposmolarity may induce adenohypophyseal secretion by producing an increase in cell volume and expansion of the outer cell membrane (1). This expansion draws secretory granules to the plasma membrane where they are extruded through some as yet undelineated exocytotic process. Conversely, contraction of cell volume induced by a hyperosmotic external environment draws the secretory granules away from the outer cell membrane and generally tends to reduce secretion. The relative rather than absolute osmolarity of the external environment appears to be the critical factor, since returning cells to a normal osmotic milieu from a hypertonic environment induces a secretory pattern very similar to that observed if the medium is changed from normal to hypotonic (1, 2). We postulated that since the dynamics of the acute response to hyposmolarity and to specific secretagogues are indistinguishable, the secretory phenomena induced by both stimuli might be exerted through a common pathway. The specific secretagogues, however, stimulate only those cells in which there is a specific receptor-activated mechanism.

In this study, we evaluated various mechanisms by which hyposmolarity might induce secretion. Ca^{2+} appears to be an essential requirement for the secretory response in many systems (7–10). However, although PRL and TSH secretion induced by TRH were markedly inhibited in a Ca^{2+}-free medium, the secretory response to hyposmolarity was actually increased by depletion of extracellular Ca^{2+}. It is possible that Ca^{2+} might be required and would be supplied by mobiliza-

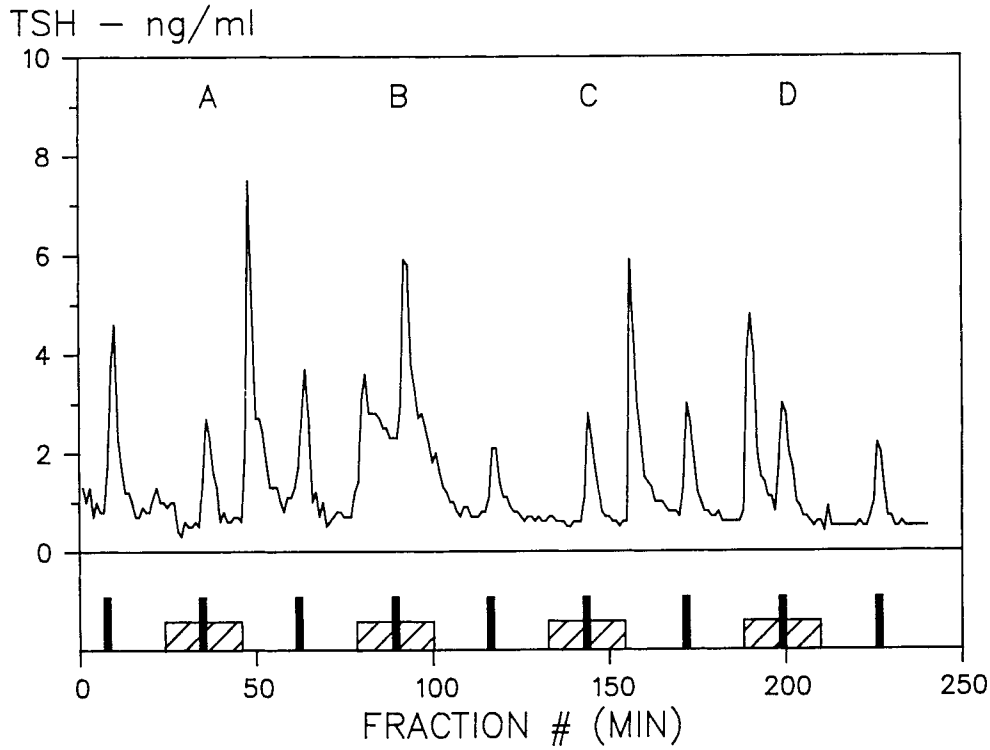

Figure 7. Acutely dispersed adenohypophyseal cells were perifused with DMEM containing 20% water to produce hyposmolarity, 2% mannitol to produce hyperosmolarity, or 100 n*M* TRH. All solid vertical black bars, except that in B, represent 2 min of 100 n*M* TRH. The hatched blocks indicate at A and C: 20 min of 2% mannitol; at B: 20 min of 100 n*M* TRH with a 2-min pulse of 2% mannitol in the middle; and at D: 20 min of 20% water.

tion from internal calcium, as has been shown for GH3 cells (11), but in the acutely dispersed cells of our studies there clearly was a difference in calcium requirements between TRH-induced stimulation and hyposmolarity-induced stimulation. Not only was the initial high-amplitude secretory burst induced by hyposmolarity increased by depletion of extracellular Ca^{2+} but the low-level "second phase" secretion of TSH and PRL seen with prolonged exposure to the same concentration was also not diminished. This contrasts with the concept that TRH-induced second phase secretion of PRL is due to influx into the cytosol of extracellular Ca^{2+} (12–14). However, this concept of the role of Ca^{2+} in secretion is largely derived from studies in tumor-derived growth hormone cells which have markedly different needs for extracellular Ca^{2+} than do normal pituitary cells (15).

The increased secretory response to hyposmolarity when $[Ca^{2+}]_e$ is reduced, although unexpected, is apparently not unique to secretion by anterior pituitary cells. Cardiac myocytes secrete atrial natriuretic peptide in response to external osmotic change. This secretory response is also significantly increased through some as yet undefined mechanism by depleting extracellular Ca^{2+} (16). Hyposmolar stimulation of renin secretion by superfused rat glomeruli is also enhanced by reducing medium Ca^{2+} (17).

Microtubules and/or microfilaments within the cell may be important constituents of movements of the plasma membrane and in migratory movements of vesicles within the cell. However, neither colchicine nor cytochalasin B had any effect on hyposmolar stimulation of secretion, suggesting that neither of these two subcellular organelles are required for this phenomenon.

Volume changes in the cell induced by alterations of external osmolarity might also be strongly linked with changes in sodium and potassium flux mediated by regulated ionic channels. However, neither ouabain nor tetrodotoxin had any effect on hyposmolar stimulation.

Only decreasing the temperature to 1°C caused a marked decrease in hyposmolar-induced secretion. This indicates that a membrane-associated phenomenon is involved since membrane mobility is markedly decreased at this temperature. However, a much more extensive exploration of temperature effects is required before any firm conclusions can be drawn.

Although the present data do not establish the mechanism by which hyposmolarity induces secretion in acutely dispersed anterior pituitary cells, they indicate that the phenomenon does not require extracellular calcium and that microtubules and microfilaments and

changes in sodium flux across the plasma membrane are not involved.

This study was supported by Research Grant DK-01447 from the NIDDK, National Institutes of Health.

We are indebted to the National Hormone and Pituitary Program, NIDDK, NIH for the reagents for TSH and PRL radioimmunoassay, Drs. Ralph Tanz, Noriyuki Sato, and Xiangbing Wang for valuable advice, Dr. Kent Thornburg and Tom Green for measurements of osmolarity, Zuzana Ophsahl and Staci McAdams for expert technical assistance, and Elizabeth Allen for her secretarial skills.

1. Greer MA, Greer SE, Opsahl Z, McCafferty L, Maruta S. Hyposmolar stimulation of *in vitro* pituitary secretion of luteinizing hormone: a potential clue to the secretory process. Endocrinology **113**:1531–1533, 1983.
2. Greer MA, Greer SE, Opsahl Z, Maruta S. Comparison of hyposmolar and hypersomolar effects on *in vitro* luteinizing hormone secretion by anterior pituitary cells. Proc Soc Exp Biol Med **178**:24–28, 1985.
3. Greer MA, Greer SE, Opsahl Z, McCafferty L, Maruta S. Hyposmolality stimulates in vitro pituitary TSH and prolactin secretion with dynamics identical to those produced by TRH. Abstracts of the 59th Meeting of the American Thyroid Association, New Orleans, pT-38, 1983.
4. Greer MA, Greer SE, Opsahl Z, Maruta S. Hyperosmolar stimulation of thyrotropin and prolactin secretion by dispersed pituitary cells: support for "cell expansion" theory of secretion. Clin Res **32**:546A, 1984.
5. Keith LD, Tam B, Ikeda H, Opsahl Z, Greer MA. Dynamics of thyrotropin-releasing hormone-induced thyrotropin and prolactin secretion by acutely dispersed rat adenohypophysial cells. Evidence for "all-or-none" secretion by heterogeneous secretory units, each with a specific response threshold. Neuroendocrinology **43**:445–452, 1986.
6. Grodsky G. A threshold distribution hypothesis for packet storage of insulin and its mathematical modeling. J Clin Invest **51**:2047–2059, 1972.
7. Marx JL. The polyphosphoinositides revisited. Science **228**:312–313, 1985.
8. Rasmussen H. The calcium messenger system. N Engl J Med **314**:1094–1101, 1986.
9. Conn PM, Huckle WR, Andrews WV, McArdle CA. The molecular mechanism of action of gonadotropin releasing hormone (GnRH) in the pituitary. Recent Prog Horm Res **43**:29–68, 1987.
10. Gershengorn MC. Mechanism of thyrotropin releasing hormone stimulation of pituitary hormone secretion. Annu Rev Physiol **48**:515–526, 1986.
11. Gershengorn MC, Thaw C, Gerry RH. Calcium influx is not required for thyrotropin-releasing hormone stimulation of prolactin release from GH3 cells. Cell Calcium **4**:117–124, 1983.
12. Aizawa T, Hinkle PM. Thyrotropin-releasing hormone rapidly stimulates a biphasic secretion of prolactin and growth hormone in GH4C1 rat pituitary tumor cells. Endocrinology **116**:73–82, 1985.
13. Albert PR, Tashjian AH Jr. Relationship of thyrotropin-releasing hormone-induced spike and plateau phases in cytosolic free Ca^{2+} concentrations to hormone secretion—Selective blockade using ionomycin and nifedipine. J Biol Chem **259**:15350–15363, 1984.
14. Gershengorn MC, Thaw C. Thyrotropin-releasing hormone (TRH) stimulates biphasic elevation of cytoplasmic free calcium in GH3 cells. Further evidence that TRH mobilizes cellular and extracellular Ca^{2+}. Endocrinology **116**:591–596, 1985.
15. Greer MA, Murakami M, Greer SE, Hjulstad S. Comparison of TRH- and hyposmolar-induced TSH and PRL secretion in cultured and acutely dispersed adenohypophysial (AP) cells. Abstracts of the 62nd Annual Meeting of the American Thyroid Association, Washington, PT42, 1987.
16. Greenwald JE, Apkon M, Hruska KA, Needleman P. Stretch-induced atriopeptin secretion in the isolated rat myocyte and its negative modulation by calcium. J Clin Invest **83**:1061–1065, 1989.
17. Skott O. Calcium and osmotic stimulation in renin release from isolated rat glomeruli. Pflugers Arch **406**:485–491, 1986.