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The Involvement of Cyclic-3',5'-AMP in the Release of Hormones from The Anterior Pituitary *in Vitro*[1] (39163)

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Recent reports have implicated cyclic-3',5'-adenosine monophosphate (cAMP) in the hormone secreting processes of the anterior pituitary. While several investigators have shown increased release of luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) in the presence of the dibutyryl derivative of cAMP (DBcAMP) (1–4), others have not been able to duplicate these results (5–8). We have previously reported (9) that cholera enterotoxin is capable of increasing pituitary cAMP levels fourfold while only potentiating hypothalamic extract (HE)-stimulated LH, FSH, and TSH release. Similar effects on TSH release were found with prostaglandins (10). In contrast, reports have been unanimous that growth hormone (GH) release can be directly stimulated *in vitro* by the addition of exogenous DBcAMP. We also observed a stimulation of GH release in association with increased cAMP concentration by the addition of cholera enterotoxin or various prostaglandins to pituitaries incubated *in vitro* (9, 10). These results suggest that a mechanism other than the adenylate cyclase system may be partially or fully responsible for the acute secretion of pituitary hormones other than growth hormone.

To further study the role of cAMP in pituitary hormone release, we have investigated the effects of agents that should increase intracellular cAMP levels, while the pituitaries were under the influence of either hypophysiotropic hormones of the hypothalamus or increased potassium ion concentrations in the incubation media. The parameters measured were pituitary hormone release and intracellular cAMP levels.

Material and methods. Hemipituitaries taken from male rats (Simonsen Laboratories, Gilroy, California) (250–300 g) were incubated for 4 hr as previously described (10). At the end of each incubation, the media were stored frozen until the hormones were measured by specific radioimmunoassays (RIA). LH was assayed by the method of Niswender *et al.* (11),[2] and the results were expressed in terms of NIH-LH-S1 (μg released per pituitary per 4 hr). FSH, prolactin (PRL), TSH, and GH were all measured using the RIA reagents generously supplied by NIAMD[2] and expressed as μg of the respective RP-1 standards.

The incubated pituitaries were immediately frozen on dry ice and lyophilized. The dry pituitaries were then weighed and extracted with 0.5 ml of 5% trichloroacetic acid for measurement of cAMP. The resulting homogenates were centrifuged and the supernatant was extracted five times with ether acidified with HCl. Cyclic-AMP was measured by the method of Gilman (12), using reagents supplied by Diagnostic Products Corp. (Culver City, Calif.). Results were expressed as picomoles of cyclic-AMP per milligram of dry pituitary.

Theophylline, adenosine-5'-monophosphate (Grade A), and adenosine (Grade A) were obtained from Calbiochem (Los Angeles, Calif.), while N^6, O^2-dibutyryl adenosine-3',5'-cyclic monophosphate (sodium salt) and guanosine-3',5'-cyclic monophosphate (sodium salt, cGMP) were obtained from Sigma Chemicals (St. Louis, Mo.). Purified cholera enterotoxin was generously supplied by Dr. R. A. Finkelstein (University of Texas Health Science Center at Dallas). A partially purified ovine hypo-

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thalamic extract was prepared by dilute acetic acid extraction followed by ultrafiltration and Sephadex gel filtration. It was found to contain significant amounts of LH-releasing hormone (LRH), FSH-releasing factor (FRF), and PRL-release inhibiting factor (PIF). Stimulation of hormone release by potassium ion was accomplished by replacing NaCl in the Krebs Ringer with an equivalent concentration of KCl. Sodium and potassium ion concentrations were confirmed by flame photometry using an I.L. model 143 flame photometer. Synthetic LRH was generously supplied by Dr. J. M. Stewart (University of Colorado Medical Center).

In each experiment, means and standard errors were determined by pooling the results from all flasks having identical treatments. Levels of significance were determined by application of Student's *t* test. The significance levels of standard treatments which produced significant alterations in every instance, such as the addition of HE, increased potassium ion, or synthetic neurohormones, have been omitted from the tables in order to avoid confusion with the significance of the experimental manipulations being studied.

Results. In order to investigate the effects of the administration of exogenous cAMP, pituitaries were incubated for 4 hr in 2 m*M* DBcAMP in the presence or absence of 1.3 mg/ml of hypothalamic extract (Table I). Dibutyryl cyclic-AMP alone significantly increased GH release 3.5-fold, although the nucleotide had no effect on either LH, FSH, TSH, or PRL release. The addition of hypothalamic extract significantly increased the release of LH, FSH, TSH, and GH while it inhibited PRL secretion. Cyclic-AMP in the presence of hypothalamic extract significantly potentiated HE-stimulated LH, FSH, and GH release while it reversed the inhibition of PRL release (Expts 1, 2). There was no further significant increase in TSH release.

The phosphodiesterase inhibitor, theophylline, might be expected to increase intracellular cAMP by virtue of its ability to inhibit phosphodiesterase. Therefore, we incubated pituitaries in 5 m*M* theophylline in the presence or absence of 1.6 mg/ml of HE (Table II, Expt 1) or 26 m*M* potassium, another stimulant of pituitary hormone release (Table II, Expt 2). The addition of theophylline alone significantly increased both GH and PRL release, while a twofold increase in intrapituitary [cAMP] was observed. There was no significant change in TSH release, and variable results were obtained with LH and FSH. The release of each gonadotropin was significantly increased in one of two experiments. Except in the case of PRL, theophylline significantly potentiated HE-stimulated and potassium ion-stimulated hormone release. In the case of PRL, the phosphodiesterase inhibitor reversed the HE-induced inhibition of PRL release. It also increased PRL release in the presence of high potassium medium. In contrast to the other hormones, elevated medium potassium failed to stimulate prolactin release.

TABLE I. EFFECT OF DIBUTYRYL cAMP ON THE *IN VITRO* RELEASE OF LH, FSH, TSH, PRL, AND GH[a]

		μg hormone release per pituitary per 4 hr				
	N	LH	FSH	TSH	PRL	GH
Expt 1						
C	4	0.20 ± 0.03	5.3 ± 0.5	28 ± 1.3	17.4 ± 0.7	33 ± 5
cAMP	4	0.19 ± 0.04	6.4 ± 0.6	28 ± 4	18.7 ± 0.4	123 ± 6**
HE	4	0.60 ± 0.04	12.1 ± 0.2	71 ± 5	10.1 ± 0.6	50 ± 3
HE + cAMP	4	1.10 ± 0.05**	17.5 ± 0.8**	98 ± 21	13.5 ± 0.6**	167 ± 7**
Expt 2						
C	8	0.14 ± 0.02	5.1 ± 0.4	23 ± 2	13.6 ± 0.7	31 ± 3
HE	8	0.52 ± 0.04	11.8 ± 0.4	49 ± 5	9.1 ± 0.5	40 ± 3
HE + cAMP	8	0.74 ± 0.04**	14.5 ± 1.0*	58 ± 8	12.6 ± 1.0*	101 ± 10*

[a] C = control; cAMP = dibutyryl cAMP, 2 m*M*; HE = hypothalamic extract 1.3, mg/ml; +cAMP = HE 1.3 mg/ml plus cAMP, 2 m*M*; *n* = number of flasks per experimental group; * = $P < 0.05$; ** = $P < 0.01$ (vs preceding flask).

In a similar set of experiments, neither adenosine, 5'-AMP, nor cGMP at the same concentrations as DBcAMP, were effective in altering the basal or stimulated release of any of the hormones studied (Sundberg, unpublished results).

In order to determine whether hormone release accompanied changes in pituitary intracellular [cAMP], we incubated glands with a variety of agents and measured hormone content of the media and intracellular cAMP levels after 4 hr of incubation (Table III). Incubation of glands in high potassium ion concentration media consistently increased the release of LH, FSH, TSH, and GH, while it had no effect on cAMP levels or release of PRL. Hypothalamic extract stimulated LH, FSH, TSH, and GH release, while it inhibited PRL release and did not alter cAMP levels. Both synthetic LRH (20 ng/ml) and TRH (100 ng/ml) elicited maximal releases of LH and TSH, respectively, although cAMP levels were unchanged. The addition of the two synthetic hormones together did produce a slight, but insignificant, elevation of cAMP. In contrast to these agents, cholera enterotoxin was found to have a dramatic stimulatory

TABLE II. EFFECT OF THEOPHYLLINE ON THE *IN VITRO* RELEASE OF LH, FSH, TSH, PRL, AND GH AND TISSUE cAMP LEVELS[a]

	N	LH[b]	FSH[b]	TSH[b]	PRL[b]	GH[b]	cAMP[c]
Expt 1							
C	4	0.13 ± 0.03	3.9 ± 0.07	19 ± 2.2	12.0 ± 0.3	27 ± 1.3	30.8 ± 2.0
Theo	4	0.16 ± 0.04	4.6 ± 0.2*	26 ± 3.1	16.5 ± 0.4*	92 ± 2.6*	67.2 ± 16.4
HE	4	0.78 ± 0.05	10.9 ± 0.5	63 ± 7.4	8.6 ± 0.6	37 ± 2.9	—
HE + Theo	4	1.15 ± 0.15*	16.2 ± 0.9*	148 ± 6.4**	14.2 ± 0.5*	126 ± 8.8*	—
Expt 2							
C	4	0.09 ± 0.01	6.2 ± 0.4	24 ± 2.0	21 ± 0.6	52 ± 5.3	—
Theo	4	0.16 ± 0.02**	7.3 ± 0.4	29 ± 2.3	30.2 ± 1.9**	178 ± 9.9**	—
K^+	4	0.24 ± 0.03	10.8 ± 1.2	87 ± 3.1	17.8 ± 1.1	232 ± 8.7	—
K^+ + Theo	4	0.62 ± 0.05**	19.2 ± 1.1**	135 ± 15.3*	27.8 ± 2.8*	332 ± 28*	—

[a] C = control; Theo = theophylline, 5 m*M*; HE = 1.6 mg/ml of the hypothalamic extract; + Theo = HE (1.6 mg/ml) plus theophylline, 5 m*M*; K^+ = 26 m*M* potassium ion concentration; K^+ + Theo = 26 m*M* potassium plus 5 m*M* theophylline. * = $P \leq .05$; ** = $P \leq .01$ (vs the preceding flask); —, not measured.

[b] μg of hormone release/pituitary/4 hr.

[c] pmoles of cAMP/mg of dry tissue.

TABLE III. INTRACELLULAR cAMP CONCENTRATIONS AND LH, FSH, TSH, PRL, AND GH RELEASE ELICITED BY A VARIETY OF BIOACTIVE AGENTS[a]

	N	cAMP[b]	LH[c]	FSH[c]	TSH[c]	PRL[c]	GH[c]
Expt 1							
C	4	43 ± 1.3	0.14 ± 0.01	5.2 ± 0.7	34 ± 5.5	9.8 ± 0.5	19 ± 2.7
HE	4	35 ± 2.2	0.88 ± 0.06*	10.8 ± 0.4*	95 ± 9.9*	8.4 ± 0.6	89 ± 6.4*
K^+ (25 m*M*)	4	41 ± 1.0	0.31 ± 0.02*	7.5 ± 0.4*	163 ± 18*	11.6 ± 0.7	195 ± 16*
K^+ (50 m*M*)	4	44 ± 3.8	0.61 ± 0.04*	13.9 ± 1.7*	281 ± 39*	9.0 ± 0.4	138 ± 3.8*
LRH (20 ng/ml)	4	48 ± 1.3	1.53 ± 0.14*	30.2 ± 3.8*	72 ± 19	10.0 ± 0.9	23 ± 0.2
TRH (100 ng/ml)	4	47 ± 3.4	0.17 ± 0.01	4.7 ± 0.4	338 ± 46*	10.0 ± 0.6	19 ± 1.5
LRH + TRH (20 + 100 ng/ml)	4	66 ± 21	1.7 ± 0.21*	37.1 ± 5.7*	327 ± 19*	12.4 ± 0.6	21 ± 8.7
Expt 2							
C	4	31 ± 2.3	0.17 ± 0.03	4.4 ± 0.2	29.0 ± 2.4	11.6 ± 0.4	52 ± 6.5
CE	4	118 ± 4.2**	0.28 ± 0.06	4.5 ± 0.2	25.0 ± 4.3	12.0 ± 0.8	114 ± 6.5*
HE	4	37 ± 4.1	1.06 ± 0.07**	12.6 ± 0.7**	95.0 ± 4.2**	8.7 ± 0.4*	87 ± 5.8
CE + HE	4	53 ± 6.5*	1.19 ± 0.06**	11.7 ± 0.5**	114.0 ± 25**	9.2 ± 0.7*	122 ± 9.2*

[a] C = control; HE = hypothalamic extract, 1.6 mg/ml; CE = cholera enterotoxin, 1 μg/ml. * = $P < 0.05$; ** = $P < 0.01$ vs control.

[b] pmoles cAMP/mg dry tissue.

[c] μg hormone released/pituitary/4 hr.

effect on cAMP levels. Nevertheless, it was effective in initiating the release only of GH. Curiously, the addition of the hypothalamic extract to glands incubated with cholera enterotoxin significantly inhibited this increase in intracellular cAMP, while eliciting release of LH, FSH, and TSH. PRL release was lowered but GH release was not altered by the addition of hypothalamic extract in the presence of the enterotoxin.

Since potassium ion-stimulated hormone release seemed to operate irrespective of cAMP concentration, glands were incubated in varying concentrations of potassium ion in the presence or absence of 1 m*M* DBcAMP (Fig. 1). Preliminary dose–response experiments showed that the synthetic releasing hormones (LRH and TRH) were capable of stimulating LH, FSH, and TSH release, respectively, to values two to three times greater than the maximal stimulation that could be accomplished by increasing the potassium ion concentration. When the potassium ion concentration reached a "threshold" of approximately 18 m*M*, release of LH and FSH was initiated (Fig. 1). In contrast, the threshold for release of GH and TSH was lower and the slope of the increase in hormone release was greater as [K^+] was increased. As before (Tables II and III), potassium ion had

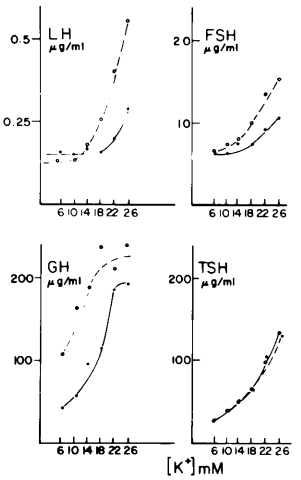

FIG. 1. Glands were incubated for 4 hr in medium containing 6, 10, 14, 18, 22, or 26 m*M* K^+ in the presence (broken line) or absence (solid line) of 1 m*M* DBcAMP. Each point represents the mean of two flasks per group.

no effect on PRL release. DBcAMP potentiated potassium ion-stimulated LH, FSH, and GH release, and the maximal release was similar to that obtained with maximal hypothalamic releasing factor stimulation. Furthermore, the nucleotide was capable of eliciting the release of FSH, LH, and GH at concentrations of potassium ion which were subthreshold for hormone release. In contrast, DBcAMP had no effect on K^+-induced TSH release.

Discussion. In this paper, we have described apparent dissociations between pituitary LH, FSH, TSH, PRL, and GH release and intracellular cAMP concentrations. In the case of LH, FSH, and TSH, the exogenous administration of DBcAMP or agents such as theophylline or cholera enterotoxin, which should increase intracellular cAMP levels in all cell types of the pituitary, was capable of stimulating hormone release only when release was stimulated by hypothalamic releasing hormones or by elevated potassium ion concentrations. Simultaneous administration of DBcAMP when the glands were stimulated by high [K^+] further increased release up to the maximum attainable with releasing hormone stimulation. While maximal release of LH, FSH, and TSH could be initiated by the addition of a hypothalamic extract or synthetic LRH and TRH, this was not accompanied by a significant increase in cAMP levels. Other reports have suggested that synthetic LRH can increase cAMP levels after a lag period of at least 90 min (13, 14) which was followed by LH release; however, we have found that LRH significantly stimulates the release of LH and FSH as early as 30 min after the addition of LRH (15). These results would suggest that increased intrapituitary cAMP concentrations did not initiate exocytosis leading to LH, FSH, and TSH release; however, cyclic-AMP does appear to induce an increase in hormone release if the exocytotic process is already initiated by the hyothalamic releasing hormones or by high potassium ion concentrations.

In contrast, GH release could be directly stimulated by exogenous dibutyryl cAMP, which also reversed the inhibition of prolactin release induced by the hypothalamic extract. The release of both of these hor-

mones was also directly stimulated by theophylline. The additive release of GH produced by high potassium and by DBcAMP would suggest that the step in the release mechanism activated by potassium ion is different from the step activated by cAMP. We have found that both GH and PRL release increases markedly when the pituitary is removed from hypothalamic influences and studied *in vitro* (15). Similar results have been reported by other gorups (16–19). This would indicate that the exocytotic process of the somatotroph and mammotroph are already operating when the glands are studied *in vitro*. Recent reports have shown that exogenous cAMP can increase protein synthesis (2) and the synthesis of LH, GH, and PRL (21) in the pituitary. Therefore, the observed direct effects of DBcAMP, theophylline, cholera enterotoxin, and prostaglandins (10) on GH release could be the result of release secondary to increased hormone synthesis rather than activation of the release mechanism.

The inhibition of cholera enterotoxin-induced cAMP accumulation by hypothalamic extract is puzzling but could possibly be explained by the presence of PIF in the extract.

In conclusion, our results suggest that elevation of intracellular cAMP does not initiate the exocytotic process, leading to pituitary hormone release. It does, however, promote hormone release when it is already stimulated. Therefore, cAMP appears to increase the availability of pituitary hormones for the secretory process by increasing either hormone synthesis (20, 21) or transport to the plasma membrane.

Summary. DBcAMP significantly increased the release of GH but not of LH, FSH, TSH, or PRL, except in the presence of hypothalamic extract when it augmented the release of LH, FSH, and GH, reversed the inhibition of PRL, but did not further influence TSH release. Theophylline increased release of GH and PRL while inducing increased tissue content of cAMP without consistently increasing the release of TSH, LH, or FSH. Hypothalamic extract or K^+-stimulated hormone release was consistently and significantly potentiated by theophylline. Neither hypothalamic extract, increased $[K^+]$, nor synthetic TRH and LRH were able to raise tissue content of cAMP while producing their expected effects on hormone release. Cholera enterotoxin produced a highly significant increase in tissue content of the cyclic nucleotide but increased the release of GH only, and not that of LH, FSH, TSH, or PRL. DBcAMP was able to lower the threshold concentration of K^+ required to stimulate release of GH, LH, and FSH and also to augment K^+-stimulated release to the higher levels induced by the hypothalamic releasing hormones. It did not augment K^+-induced release of TSH.

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