STUDIES ON GROWTH HORMONE SECRETION: III. INHIBITION OF PROSTAGLANDIN, THEOPHYLLINE AND CYCLIC AMP STIMULATED GROWTH HORMONE RELEASE BY VALINOMYCIN IN VITRO

F. Hertelendy,* G. Peake** and H. Todd*

*New Enterprise Division, Monsanto Co., St. Louis, Mo. 63166

**University of New Mexico, School of Medicine, Albuquerque, New Mexico

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SUMMARY

Rat anterior pituitary glands were incubated in medium 199 or KRB and the secretion of growth hormone was measured by immunoassay. Prostaglandin E_1, dibutyryl cyclic AMP, theophylline and K^+ stimulated growth hormone release. Valinomycin inhibited hormone responses to all these stimuli without depressing intracellular cyclic AMP levels. Stimulation of growth hormone secretion by dibutyryl cyclic AMP and theophylline was associated with increased oxidation of glucose-1-C^{14} to $C^{14}O_2$ which was also inhibited by valinomycin. It is suggested that the locus of the inhibition is distal to the generation of cyclic AMP and might be analogous to the inhibitory effect of dinitrophenol.

Kuo and Dill (1) reported that the antibiotic valinomycin inhibited epinephrine stimulated lipolysis in fat cells and decreased formation of intracellular cAMP-8-^{14}-C from adenine-8-^{14}C. They attributed the valinomycin induced inhibition of lipolysis to the apparent decrease in adenyl cyclase activity. However, caffeine and theophylline were found ineffective in reversing the valinomycin block to lipolysis. Subsequently, Bentley (2) demonstrated that valinomycin antagonized the effects of vasopressin and aldosterone, but not of exogenous cAMP, on the toad bladder. He interpreted his data to indicate that valinomycin inhibited adenyl cyclase, since the action of both these hormones is believed to be mediated by cAMP. Since neither of these two groups of investigators measured the intracellular concentration of

cyclic AMP, the precise locus of valinomycin inhibition of cAMP mediated cellular events was not clarified.

In this communication evidence is presented which shows that valinomycin inhibits cAMP (endogenous or exogenous) mediated GH release from rat anterior pituitaries in vitro suggesting a site of action distal from the generation of cAMP.

MATERIALS AND METHODS

Male Charles River rats (200-230 gm) maintained on standard Purina laboratory chow were decapitated and the anterior pituitaries dissected out, rinsed in saline and incubated in either medium TC199 or KRB (pH 7.4) containing glucose (1 mg/ml) and BSA (1 mg/ml) in an atmosphere of O_2:CO_2 (95:5) with constant shaking. More detailed descriptions of the experimental conditions are given in the appropriate headings to the tables.

Growth hormone in the media, which was frozen on dry ice, immediately at the end of incubation, was determined by radioimmunoassay (3). For cAMP determination the tissues were frozen in liquid nitrogen and kept at -80°. Before extraction and analysis by the radioimmunoassay method of Steiner et al. (4), the glands were weighed and tissue concentrations of cAMP are expressed on the basis of these weights.

Glucose-1-C^{14} oxidation to $C^{14}O_2$ was measured by incubating one whole anterior pituitary gland (average weight 6.62 ± 0.20 mg, range 5.15-8.10) in 2 ml KRB for three hours. The 10 ml Erlenmeyer incubation flasks were fitted with rubber stoppers and plastic cups for the collection of CO_2. The flasks were gassed with O_2:CO_2 (95:5) for 10 minutes and incubated at 37° in a metabolic shaker. At the end of the incubation period, 0.20 ml of medium was withdrawn for GH assay followed by the injections of Hyamine (0.25 ml) into the cup and 3N H_2SO_4 (0.25 ml) in the flask. After shaking

for an additional one hour, the plastic cups were transferred to scintillation vials containing 15 ml of Packard Insta-Gel cocktail and counted in a Nuclear Chicago Mark I scintillation counter. Values, corrected for quenching, are expressed as cpm/mg pituitary/ 3.5 hr.

RESULTS AND DISCUSSION

The results in Table 1 confirm the GH releasing potency of theophylline and dcAMP. They also demonstrate that PGE_1 is a potent stimulant of GH release *in vitro* as measured directly by immunoassay. Recently MacLeod and Lehmeyer (5) reported that PGE_1 increased the incorporation of labeled leucine into pituitary GH as well as the radioactivity of GH in the medium. These data, together with the observations that PGE_1 elevated cAMP levels in the pituitary (6), thyroid (7) and in a variety of other tissues (8) strongly suggest that its hormone releasing effect is mediated via the adenyl cyclase-cAMP system.

The finding (Table 1) that valinomycin inhibited both PGE_1 and theophylline stimulated GH release supports the notion that the antibiotic acts at the adenyl cyclase level. However, the fact that valinomycin also effectively inhibited GH response to exogenous dcAMP suggests a different site of action: one that is involved with the action of the second messenger cAMP rather than with the formation of it.

This concept is further strengthened by the data presented in Table 2 which show that valinomycin while completely abolishing theophylline stimulated release of GH failed to inhibit the intracellular accumulation of cAMP and when added to the incubation media alone caused a significant rise (143%) in cAMP concentration.

Table 1. Inhibition by valinomycin of growth hormone responses to prostaglandin E_1 dibutyryl-cyclic AMP and theophylline

	Additions	GH secretion rate µg/mg tissue/60 min	P	
A	None	0.82 ± 0.03	A vs B	<.01
B	Valinomycin (10^{-5} M)	0.59 ± 0.04	A vs C	<.001
C	Prostaglandin E_1 (5×10^{-5} M)	4.92 ± 0.18	C vs D	<.001
D	PGE_1 + Valinomycin	2.62 ± 0.25	A vs E	<.001
E	Dibutyryl-cyclic AMP (2×10^{-3} M)	1.91 ± 0.09	E vs F	<.001
F	Dibutyryl-cAMP + Valinomycin	1.07 ± 0.08	A vs G	<.001
G	Theophylline (2.5×10^{-3} M)	2.47 ± 0.12	G vs H	<.001
H	Theophylline + Valinomycin	0.76 ± 0.11		

Four half pituitaries were preincubated in 1 ml TC 199 per flask for 1 hr. followed by a second 1 hr. "experimental" period in fresh medium containing the appropriate additions. Valinomycin was dissolved in acetone and PGE_1 in EtOH. Control flasks received appropriate volumes of solvents. There were 5 flasks/group with the exception of C and D which consisted of 6 replicates each. Values are mean ± SE of duplicate determinations of 2 different dilutions. Statistical comparisons by the t test.

Table 2. Effect of valinomycin and theophylline on cyclic AMP levels in rat anterior pituitaries and on the release of growth hormone

Additions	cAMP nmole/g	GH µg/mg/30 min
None	0.32 ± .01	0.88 ± .09
Val (10^{-5} M)	0.74 ± .07	0.74 ± .05
Theo ($5x10^{-3}$ M)	1.31 ± .22	1.65 ± .16
Theo + Val	1.08 ± .07	0.85 ± .05

Three whole anterior pituitaries/flask were incubated in 1 ml TC 199 for 30 min. without preincubation. All vessels contained acetone (5 µl). Values represent means of 5 observations ± SE.

The inhibition of GH release by valinomycin is not overcome by high Ca^{++} (5 x control) or by high K^+ (10 x control) concentrations. Omission of Ca^{++} or K^+ from the medium or raising Ca^{++} concentration had no effect on basal release of GH. These results do not, of course, exclude the possibility that valinomycin may exert its inhibitory effect by interferring with transmembrane flux of ions and/or with the intracellular translocation of ions. High K^+ medium, as expected, caused a very significant increase in GH release. It is of particular interest that K^+, which appears to be a non-specific stimulus of pituitary hormone release, does not affect intracellular cAMP levels (9), suggesting that its hormone releasing action is not dependent on increased adenyl cyclase activity. Thus, the inhibition of the K^+ effect by valinomycin (Table 3) further indicates that the site of action of the antibiotic is not at the adenyl cyclase level.

Table 3. Effect of high Ca^{++} and K^{+} concentrations on the inhibition by valinomycin of growth hormone release

	Treatment	Number of Observations	GH in Medium µg/mg/hr	P	
A	KRB	5	1.45 ± .10	A vs B	NS
B	High Ca^{++} (6.35×10^{-3} M)	3	1.78 ± .35	A vs C	NS
C	Ca^{++} Free KRB	6	1.77 ± .12	A vs D	<.02
D	Valinomycin (10^{-5} M)	6	1.02 ± .14	A vs E	<.001
E	Theophylline (5×10^{-3} M)	6	3.58 ± .27	E vs F	<.001
F	Val + Theo	5	1.50 ± .14	F vs G	NS
G	Val + Theo + High Ca^{++}	6	1.29 ± .08	A vs H	NS
H	K^{+} Free KRB	6	1.66 ± .29	H vs I	<.05
I	K^{+} Free KRB + Val	6	0.82 ± .16	A vs J	<.001
J	High K^{+} (5.4×10^{-2} M)	5	4.78 ± .42	J vs K	<.001
K	High K^{+} + Val	6	2.93 ± .12		

One hemi-pituitary was preincubated for 1 hr. in "normal" KRB (1.27 mM Ca^{++}) followed by a second 1 hr. period with the appropriate additions. In the Ca^{++} and K^{+} free experiments the tissue pieces were preincubated in media from which these cations were omitted. The osmolarity of KRB was maintained by substituting Na^{+} for Ca^{++} or K^{+}. Values represent means of the number of observations indicated ± SE. Statistical comparisons by the "t" test.

Preliminary studies (Table 4) demonstrate that in the present system dcAMP and theophylline stimulate glucose oxidation via the pentose shunt contrary to their action in adipose tissue where both these compounds inhibit the incorporation of glucose-1-C^{14} into CO_2 (10,11). The stimulation of glucose oxidation by dcAMP and theophylline was completely inhibited in this study by valinomycin. A parallel inhibition of dcAMP and theophylline stimulated GH secretion by valinomycin was, once again, clearly demonstrated.

Since valinomycin is a potent inhibitor of oxidative phosphoryla-

Table 4. Effect of dibutyryl-cyclic AMP, theophylline and valinomycin on glucose-1-C^{14} oxidation to CO_2 in rat anterior pituitaries

Addition	N	$C^{14}O_2$ cpm/mg/AP/3.5 hr	Growth Hormone μg/mg/AP/3.5 hr
None	5	460 ± 22	4.54 ± 0.19
Valinomycin (10^{-5} M)	4	463 ± 47	4.84 ± 0.40
dcAMP (2×10^{-3} M)	4	662 ± 51	10.55 ± 0.61
dcAMP + Valinomycin	4	444 ± 45	5.29 ± 0.48
Theophylline (5×10^{-3} M)	4	685 ± 56	12.08 ± 2.45
Theophylline + Valinomycin	4	330 ± 31	5.89 ± 0.19

One whole anterior pituitary was incubated in 2 ml KRB containing 0.1 mg/ml glucose (0.25 μci G-1-C^{14}) and 1 mg/ml of BSA. For growth hromone assay 0.2 ml aliquot of media were withdrawn with a Hamilton syringe prior to the injection of acid to the incubation vessels. CO_2 counts have been corrected accordingly. All values represent means ± SEM.

tion and an activator of ATPase in rat liver mitochondria (12), its action might be analogous to that of DNP which also inhibits basal as well as PGE_1 and theophylline stimulated GH secretion (13). It appears quite conceivable, therefore, that valinomycin elicits its inhibitory effect on GH secretion by primarily interferring with the energy metabolism of the pituitary cells.

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