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In GH_3 Pituitary Cells, Acetylcholine and Vasoactive Intestinal Peptide Antagonistically Modulate Adenylate Cyclase, Cyclic AMP Content, and Prolactin Secretion

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SUMMARY

In GH_3 pituitary cell homogenates, acetylcholine (ACh) (IC_{50} 200 nM) inhibits adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity in a concentration- and GTP-dependent manner. Maximal inhibition was obtained with 10 μM ACh and corresponded to approximately a 50% decrease in basal enzyme activity. ACh inhibition is antagonized by atropine and is mimicked by muscarinic receptor agonists, but not by nicotine. ACh reduces the adenylate cyclase stimulation by vasoactive intestinal peptide (VIP), without changing its EC_{50}. In intact GH_3 cells, ACh decreases the cyclic AMP content and the rate of prolactin release in a concentration-dependent manner. When the cells are simultaneously exposed to VIP and ACh, the VIP-induced increases in cyclic AMP accumulation and prolactin release are reduced by 80% and 40%, respectively. The potency of VIP is not significantly changed by the presence of ACh, and vice versa.

INTRODUCTION

In anterior pituitary, cyclic AMP functions as one second messenger (1) in PRL[2] secretion. VIP, an endogenous PRL-releasing factor (2), stimulates pituitary adenylate cyclase activity (3–5). Exposure of rat anterior pituitary cells to cyclic nucleotide phosphodiesterase inhibitors (6, 7) or cholera toxin (6)[3] increases the cyclic AMP content and the rate of PRL release. On the other hand, dopamine, which decreases PRL release (9, 10), inhibits adenylate cyclase activity and cyclic AMP accumulation in anterior pituitary (5, 7, 11, 12).

The rat GH_3 pituitary cell line, which secretes PRL (13), lacks functional dopamine receptors (14) but possesses VIP and muscarinic receptors. When activated, VIP receptors stimulate cyclic AMP production and PRL release (15), while muscarinic receptors inhibit PRL release (16). Analogues of cyclic AMP (17), phosphodiesterase inhibitors (15, 17), and cholera toxin (18) can increase PRL release from GH_3 cells. In the present study we investigated whether GH_3 muscarinic receptors are coupled with adenylate cyclase and whether these receptors interact with those for VIP in controlling the rates of cyclic AMP production and PRL secretion.

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[2] The abbreviations used are: PRL, prolactin; VIP, vasoactive intestinal peptide; ACh, acetylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; IBMX, 1-methyl-3-isobutylxanthine; PBS, phosphate-buffered saline.

[3] P. Onali, J. P. Schwartz, and E. Costa, in preparation.

EXPERIMENTAL PROCEDURES

Materials. [α-^{32}P]ATP (30–40 Ci/mmole) was obtained from Amersham (Arlington Heights, Ill.). [2,8-^{3}H]Cyclic AMP (25 Ci/mmole) was obtained from New England Nuclear Corporation (Boston, Mass). VIP was purchased from Boehringer-Mannheim (Indianapolis, Ind.) and Peninsula Laboratories (San Carlos, Calif.). All of the other compounds were from Sigma Chemical Company (St. Louis, Mo.). The cyclic nucleotide phosphodiesterase inhibitor IBMX was obtained from Calbiochem (La Jolla, Calif.). Culture media and sera were purchased from Grand Island Biological Company (Grand Island, N. Y.).

Cell culture. The rat GH_3 pituitary cell line (CCL 82.1) was obtained from the American Type Culture Collection (Rockville, Md.) and grown as monolayer cultures in Ham's F-10 medium supplemented with 2.5% fetal calf serum, 15% horse serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37° in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every 2–3 days and 24 hr before the experiments. Cells were used 6–9 days after plating.

Adenylate cyclase assay. Before each experiment, the medium was removed, and the dishes (100 × 20 mm) were rinsed with 20 ml of PBS. Cells were scraped from dishes in ice-cold homogenizing buffer [5 mM Tris-HCl (pH 7.5)/1 mM dithiothreitol/1 mM EGTA, 0.5 ml/dish] and kept in this hypotonic medium for 5–10 min at ice-bath temperature. Cells were then lysed with a Dounce tissue grinder (pestle B, 15 strokes), and the homogenate was centrifuged at 1000 × *g* for 30 sec. The supernatant was used immediately for the enzyme assay.

The adenylate cyclase activity was assayed in a 150 μl of reaction mixture containing 75 mM Tris-HCl (pH 7.4), 0.5 mM [α-^{32}P]ATP (60–80 cpm/pmole), 2 mM $MgCl_2$, 1 mM cyclic AMP, 0.5 mM IBMX, 5 mM phosphocreatine, creatine phosphokinase (50 units/ml), 0.1 mM GTP, 50 μg of bovine serum albumin, 0.33 mM EGTA, 0.33 mM dithiothreitol, 10 μM eserine, and 50–80 μg of cell protein. Bacitracin (10^{-5} M) was included in the assay mixture when VIP was present. The reaction was initiated by the addition of the cell homogenate and carried out at 37° for 10 min. The incubation was stopped by adding 200 μl of a solution

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containing 2% (w/v) sodium dodecyl sulfate, 45 mM ATP, and 1.3 mM cyclic AMP (pH 7.5). Following the addition of [2,8-^{3}H]cyclic AMP (10–15 × 10^3 cpm) to monitor the cyclic AMP recovery, the samples were placed in a boiling water bath for 3 min. Cyclic AMP was isolated as described by Salomon *et al.* (19). Protein content was determined by the method of Bradford (20), using bovine serum albumin as a standard.

Cyclic AMP assay. The cells (in 60 × 15 mm Petri dishes) were incubated in 2.7 ml of serum-free Ham's F-10 medium (without $NaHCO_3$) containing 10 mM Hepes (pH 7.4), 10 μM eserine, 10 μM bacitracin, and 0.1% bovine serum albumin. The medium was adjusted to isoosmolarity by the addition of NaCl. After a preincubation for 60 min with 0.2 mM IBMX at 37°, 300 μl of medium containing the drug being tested were added. Ten minutes later the incubation was terminated by removing the medium, washing once with 2 ml of PBS, and adding 1 ml of 0.3 N HCl to extract cyclic AMP (10 min at room temperature). The HCl extract was lyophilized and diluted in 50 mM NaAc (pH 4) to measure cyclic AMP (21). The cell residue was solubilized in 1 N NaOH and the protein content was determined according to the method of Lowry *et al.* (22), using bovine serum albumin as the standard. A 10-min incubation with drugs was chosen because cyclic AMP levels had reached a steady state by that time.

Measurement of prolactin secretion. All of the experiments were performed in triplicate using cells grown in Petri dishes (35 × 10 mm) in an air/CO_2 incubator at 37°, in serum-free Ham's F-10 medium containing 10 mM Hepes (pH 7.4), 10 μM eserine, 10 μM bacitracin, and 0.1% bovine serum albumin. After 10 min of preincubation the medium was replaced with 1 ml of medium with or without the drugs being tested, and prolactin was assayed in the medium following 60 min of incubation. The rate of release was linear for at least 2 hr, regardless of drug treatment. One hour of incubation was chosen to give amounts of PRL that were readily assayable given the sensitivity of the assay. Absolute prolactin levels varied from experiment to experiment but were consistent within a given experiment.

The kit for the prolactin radioimmunoassay was provided by Dr. S. Raiti, National Pituitary Agency, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases). Samples were assayed in triplicate.

Statistics. Significance was determined by Student's *t*-test. All EC_{50} and IC_{50} values were obtained by log probit analysis.

RESULTS

ACh inhibited the adenylate cyclase activity of GH_3 cell homogenates in a concentration-dependent manner (Fig. 1). A maximal inhibition (50% decrease) was elicited by 10 μM ACh. The ACh concentration which produced half-maximal inhibition (IC_{50}) was 200 nM. The attenuation of adenylate cyclase activity elicited by ACh was due to a decrease in the GTP-dependent enzymatic activity (Fig. 2). In the absence of GTP, ACh failed to inhibit the adenylate cyclase, whereas the inhibition increased with increasing concentrations of GTP.

Various muscarinic cholinergic receptor agonists, at equimolar concentrations, attenuated the adenylate cyclase activity of GH_3 cells by the same degree (Table 1). Nicotine, however, was completely ineffective. Furthermore, atropine antagonized the adenylate cyclase inhibition by 10 μM ACh in a dose-dependent fashion; 20 nM atropine reversed by 50% the inhibition of adenylate cyclase, whereas 1 μM completely blocked this inhibition. In contrast, *d*-tubocurarine (10 μM), a nicotinic receptor blocker, failed to antagonize the ACh inhibition (results not shown).

ACh also antagonized the adenylate cyclase activation by VIP. VIP stimulated the enzyme activity in a concentration-dependent manner with an apparent EC_{50} (concentration which caused half-maximal stimulation) of 20

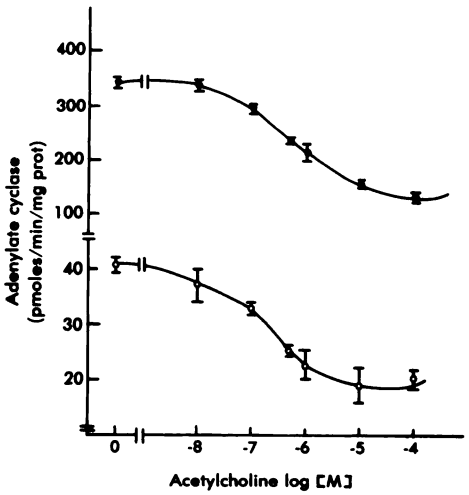

FIG. 1. *Concentration-dependent inhibition of VIP-stimulated and basal adenylate cyclase by ACh*

Adenylate cyclase activity was assayed in the presence of the indicated concentration of ACh without (○) and with (●) 20 nM VIP. Values for the VIP-stimulated activity are the means ± standard deviation of triplicate determinations from one experiment which was confirmed by an additional experiment. Values for the basal activity are the mean ± standard error of the mean of three separate experiments performed in triplicate.

nM (Fig. 3A). Maximal activation (11-fold increase) was reached at a concentration of 500 nM. In the presence of 10 μM ACh, the stimulation of adenylate cyclase by VIP was reduced by about 60%, whereas ACh failed to change the EC_{50} dose of VIP. Thus, when muscarinic receptors were occupied by ACh, the adenylate cyclase became less responsive to VIP stimulation. ACh inhibited in a concentration-dependent manner the adenylate cyclase stimulated by VIP, and the ACh potency (IC_{50} 400 nM) resembled that for inhibition of the basal adenylate cyclase activity (Fig. 1). Although the inhibitory potency of ACh failed to change when tested in the presence of VIP, the extent of ACh inhibition (calculated as the decrease

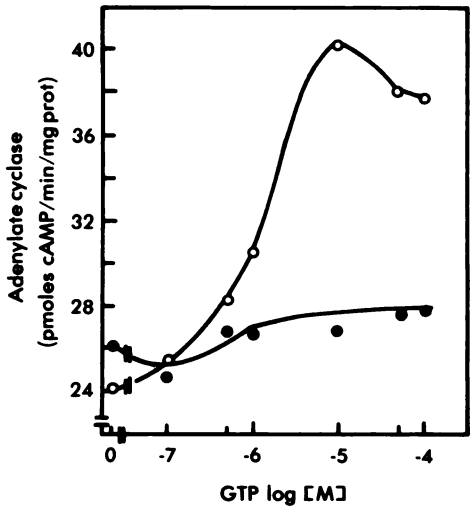

FIG. 2. *GTP dependency of adenylate cyclase inhibition by ACh*

The enzyme activity was assayed in the absence (○) and in the presence (●) of 10 μM ACh. Values are the means of triplicate determinations from one experiment with a range of variability less than 10%.

TABLE 1

Effect of various cholinergic agonists on basal adenylate cyclase, basal cyclic AMP and basal prolactin secretion in GH_3 cells

Adenylate cyclase activity was assayed in the absence and in the presence of the indicated cholinergic agonists at a concentration of 10 μM. Intact cells were incubated with the indicated concentrations of drugs for 10 min (cyclic AMP) or 60 min (prolactin). Values are the means ± standard error of the mean of the number of determinations reported in parentheses.

Addition	Adenylate cyclase	Cyclic AMP	Prolactin
	pmoles cyclic AMP/mg protein/min	*pmoles/mg protein*	*ng/mg protein/hr*
Vehicle	23.9 ± 0.5 (4)	133.2 ± 11.4 (5)	17.1 ± 0.8 (3)
Acetylcholine	13.8 ± 0.6[a] (4)	66.4 ± 4.2[b] (6)	8.6 ± 0.1[b] (3)
Oxotremorine	13.7 ± 0.2[a] (3)	86.3 ± 2.5[b] (3)	9.4 ± 0.8[b] (3)
Carbachol	14.0 ± 0.6[a] (3)		
Methacholine	14.4 ± 0.5[a] (3)		
Nicotine	23.2 ± 0.4 (3)	132.4 ± 9.6 (3)	19.3 ± 1.8 (3)

[a] $p < 0.001$.
[b] $p < 0.01$.

in the number of picomoles of cyclic AMP formed by the cyclase) increased when the enzyme was stimulated by VIP.

To ascertain whether the interaction between VIP and ACh receptors at the level of the adenylate cyclase system had any effect on the cyclic AMP content of intact GH_3 cells, we measured the intracellular content of cyclic AMP following a brief incubation with both neurotransmitters. An incubation period of 10 min was chosen because the effects of the neurotransmitters on cyclic AMP were maximal at this time. VIP increased the cyclic AMP content of GH_3 cells 9-fold, with an EC_{50} of 10 nM and maximal stimulation at 100 nM (Fig. 3B). In the presence of 100 μM ACh, the elevation of cyclic AMP in response to each concentration of VIP was inhibited by approximately 70%, whereas the EC_{50} value of VIP was not changed. Moreover, the IC_{50} values for ACh in reducing basal cyclic AMP (Fig. 4B) and in curtailing the increase in cyclic AMP content elicited by a maximally effective VIP concentration (100 nM) (Fig. 4A) are comparable (150 nM and 300 nM, respectively).

If cyclic AMP is the second messenger for the regulation of PRL release by VIP and ACh, then ACh-elicited changes in the cyclic AMP content should go *pari passu* with changes in PRL secretion from GH_3 cells. The results reported in Fig. 5 show that inhibition of both basal and VIP-stimulated PRL secretion occurred at similar concentrations of ACh (IC_{50} 200 nM and 700 nM, respectively). The EC_{50} for VIP was not changed in the presence of ACh (500 pM for basal versus 460 pM with ACh). Incubation in the presence of 0.2 mM IBMX resulted in a higher basal rate of secretion: the relative effects of VIP and ACh were not changed.

The data in Fig. 6 show that the potency of ACh in inhibiting basal PRL release correlates with its ability to inhibit adenylate cyclase and with its ability to reduce the cyclic AMP content of the cells: comparable inhibition of all of these parameters occurs at any given concentration of ACh. Furthermore, all three effects are mediated by a receptor with muscarinic properties (Table 1). However, there is a disparity between the concentration of VIP effective in stimulating PRL release (EC_{50} 500 pM) and in increasing cyclic AMP (EC_{50} 10 nM) (Fig. 7A). The EC_{50} for VIP in stimulation of adenylate cyclase (20 nM) is comparable to that for cyclic AMP. In spite of these differences, ACh inhibits the VIP stimulation of

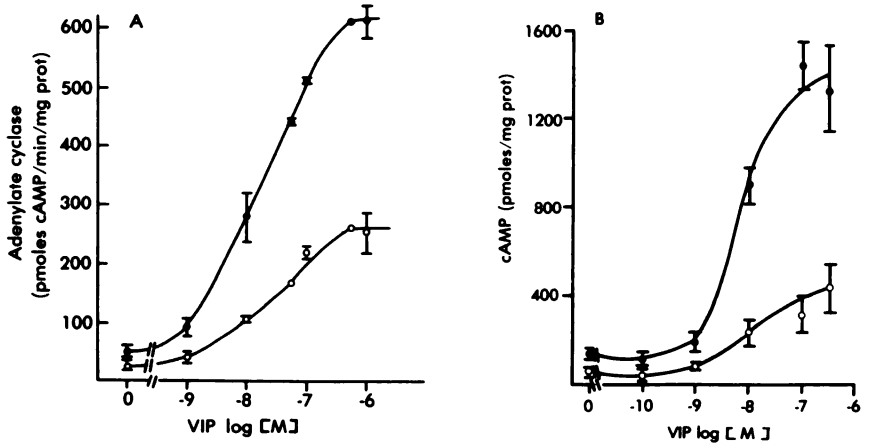

FIG. 3. *Dose-dependent stimulation by VIP*

A. Dose-dependent stimulation by VIP of adenylate cyclase in the absence or presence of acetylcholine. Enzyme activity was assayed under the conditions described under Experimental Procedures in the presence of the indicated concentrations of VIP without (●) and with (○) 10 μM ACh. Values are the means ± standard error of the mean of three experiments.

B. Dose-dependent stimulation by VIP of the cyclic AMP content of GH_3 cells in the absence or presence of ACh. Dishes of cells were incubated for 10 min with the indicated concentrations of VIP without (●) or with (○) 100 μM ACh. The calculated EC_{50} values of VIP are approximately 8 nM and 20 nM, respectively. Each point represents the mean ± standard error of the mean (n = 2–4 dishes) from one experiment representative of two experiments.

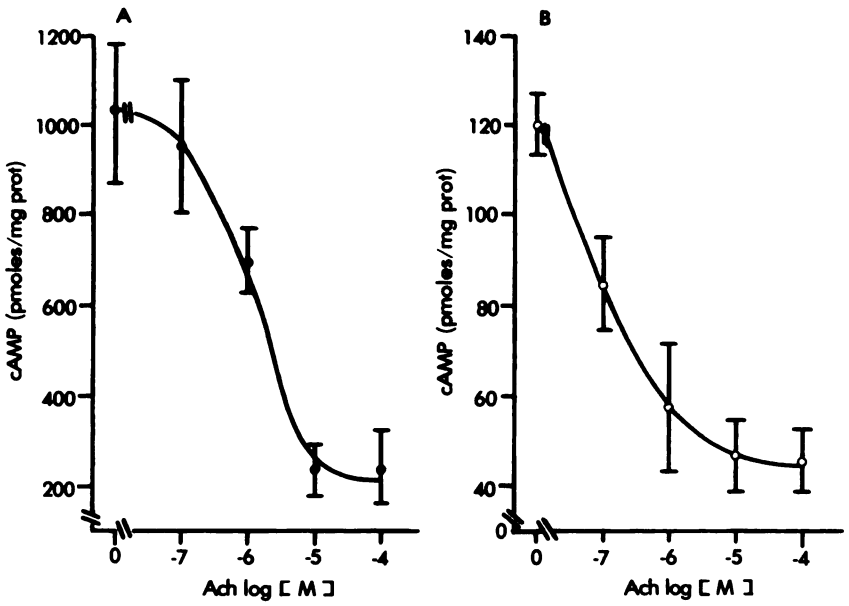

FIG. 4. *Dose-dependent inhibition by ACh*

A. Dose-dependent inhibition by ACh of VIP stimulation of cyclic AMP levels. The IC_{50} was 300 nM. Each point represents the mean ± standard error of the mean (n = 3 dishes) from one experiment, repeated twice.

B. Dose-dependent decrease by ACh of basal cyclic AMP levels. The IC_{50} was 150 nM. Each point represents the mean ± standard error of the mean (n = 3) of one experiment, repeated twice.

both PRL release and cyclic AMP content at every dose of VIP (Fig. 7B). These results support the hypothesis that ACh inhibits PRL release through its inhibition of adenylate cyclase and concomitant lowering of cyclic AMP levels.

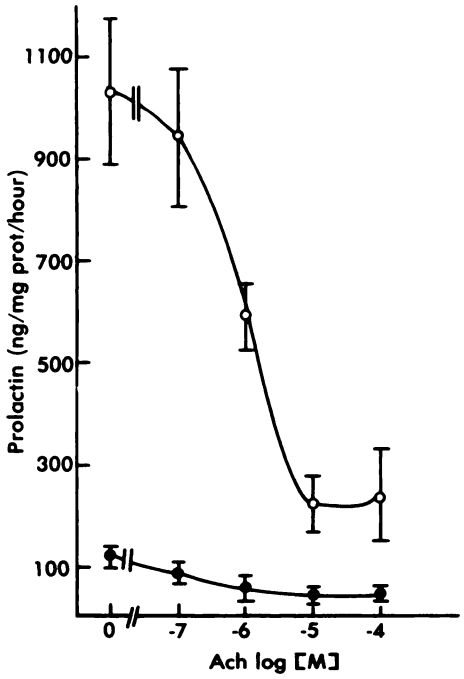

FIG. 5. *Concentration-dependent inhibition of VIP-stimulated and basal prolactin release by ACh*

Dishes of cells were incubated for 1 hr under basal (●) conditions or with 100 nM VIP (○) in the presence of the indicated concentrations of ACh. Prolactin was measured in the medium as described under Experimental Procedures. Each point represents the mean ± standard error of the mean (n = 3 dishes): the experiment was repeated twice.

DISCUSSION

Available evidence indicates that a positive correlation exists between the rate of cyclic AMP accumulation and the extent of PRL secretion in both anterior pituitary and in GH_3 cells. This relationship suggests that the recognition sites of various neurotransmitters which control PRL release may be coupled to adenylate cyclase. Previous investigations from our laboratory and other laboratories have shown that dopamine, an endogenous inhibitor of PRL secretion, attenuates the VIP-mediated activation of adenylate cyclase in rat anterior pituitary mammotrophs (5, 7, 11, 12). In the present study we show that, in GH_3 cells, ACh, another neurotransmitter which inhibits PRL release, reduces both basal and VIP-stimulated adenylate cyclase activity. This inhibition is me-

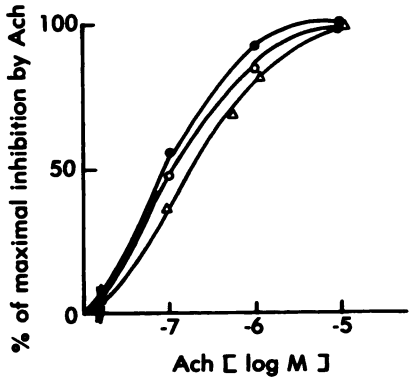

FIG. 6. *Correlation between the inhibition of adenylate cyclase, the decrease in cyclic AMP content, and the inhibition of PRL secretion elicited by ACh*

Values are expressed as percentage of the maximal inhibition at each concentration of ACh for adenylate cyclase (△), cyclic AMP (○), and PRL (●). The data used in these calculations were obtained from Figs. 1, 4B, and 5, respectively.

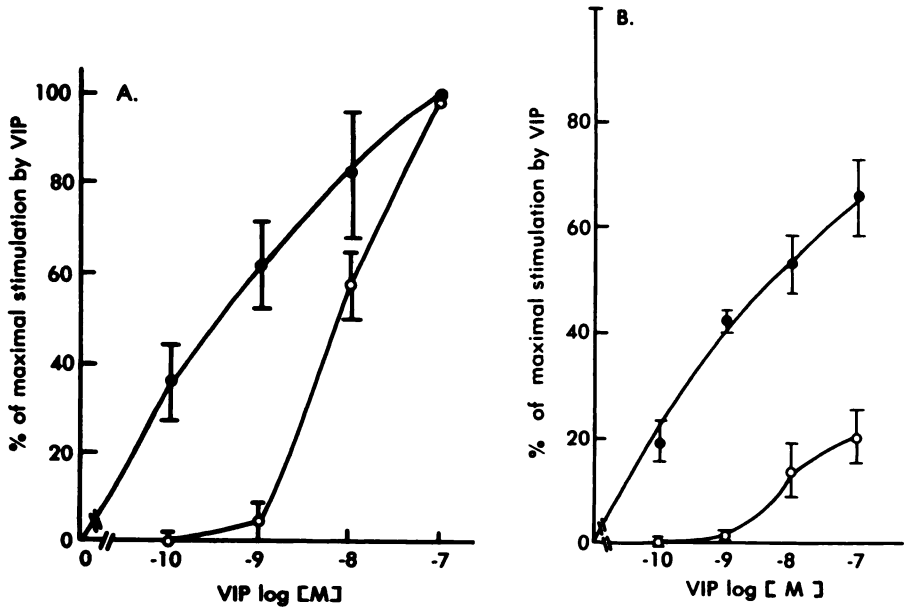

FIG. 7. *Correlation between the effects of VIP on PRL release and cyclic AMP content in the absence (A) or presence (B) of ACh*

A. Values are expressed as percentage of maximal stimulation at each concentration of VIP for PRL release (●) and cyclic AMP (○). PRL secretion was 216 ± 8 ng/mg of protein per hour (basal); 730 ± 75 (100 nM VIP). Cyclic AMP was 140 ± 9 pmoles/mg of protein (basal); 1465 ± 125 (100 nM VIP).

B. ACh (10 μM) was present in all samples. Values are expressed as percentage of maximal stimulation by VIP in the absence of ACh for PRL (●) and for cyclic AMP (○). In the presence of 100 nM VIP plus 10 μM ACh, PRL release was 478 ± 37 ng/mg of protein per hour, and cyclic AMP was 338 ± 68 pmoles/mg of protein.

diated through an action on muscarinic recognition sites, and in intact GH_3 cells is expressed by a diminution of the basal cyclic AMP content as well as of the accumulation in response to VIP. We have also shown that the ACh concentrations eliciting these effects on the cyclic AMP-generating system are comparable to those which suppress the spontaneous and VIP-stimulated PRL release. Furthermore, changes in cyclic AMP content precede changes in PRL release. Thus, these findings suggest that in GH_3 cells the reduction of PRL release elicited by cholinergic agonists is due to the inhibitory coupling of the muscarinic recognition sites with the adenylate cyclase.

The inhibitory constraint exerted by the muscarinic receptors on the adenylate cyclase is amplified when the enzyme is activated by VIP. The activity of adenylate cyclase in the presence of VIP plus ACh is less than that expected from the algebraic sum of the activities in the presence of each neurotransmitter individually (Figs. 1 and 3A). A similar pattern was also observed with respect to cyclic AMP accumulation and PRL release (see Figs. 3B, 4, and 5). In each of the responses studied, the simultaneous presence of ACh and VIP was characterized by a reduction in the efficiency of VIP which coincided with an amplification of the inhibitory response to ACh but with no change in the direction of the response.

This type of interaction can be explained by assuming that in GH_3 cells the receptors for VIP and those for ACh control the same "pool" of adenylate cyclase, probably acting through separate guanine nucleotide-binding proteins (G/F protein). As previously shown with other tissues (23–25), the attenuation of adenylate cyclase activity is dependent on the presence of GTP, suggesting that muscarinic receptors are coupled to a regulatory protein(s) that binds GTP (inhibitory G/F protein). Activation of this inhibitory coupling protein would reduce the rate of cyclic AMP formation by the catalytic subunit of adenylate cyclase. This hypothesis is supported by studies on striatal muscarinic receptors, which are also coupled in an inhibitory manner to adenylate cyclase (25) and which stimulate a high-affinity GTPase activity which may be associated with the inhibitory G/F protein(s) (26).

When the enzyme has been stimulated by VIP, probably through a stimulatory G/F protein, the activated cyclase can be inhibited by coupling with muscarinic recognition sites, and the reduction in enzyme activity elicited by ACh is greater than that in the absence of VIP. The system thus may allow a synchronous increase in the inhibitory input as more enzyme is activated by VIP, with a consequent decrease in the intracellular levels of cyclic AMP and a reduction in the amount of PRL secreted in response to the neuropeptide. However, ACh does not inhibit completely the basal activity of the cyclase nor the stimulation of the cyclase by VIP. This limitation could be due to various factors, such as an imbalance between the number of G/F inhibitory and stimulatory proteins, a non-homogeneous distribution of the muscarinic receptors in each cell, or some other condition that would cause the presence of cyclase activity insensitive to ACh inhibition. This limitation in the extent of enzyme inhibition is reflected in the partial decrease of cyclic AMP content and the partial inhibition of PRL release elicited by ACh.

Certain evidence indicates that cyclic AMP may not be the only second messenger involved in the regulation of PRL secretion. Recent work in the field, summarized by Dannies (27), suggest that PRL processing and secre-

tion are not homogeneous processes and that any particular hormone may affect more than one step. The data presented in Fig. 7A, in agreement with previous results (28), show that VIP can increase PRL release at doses which do not increase cyclic AMP and therefore suggest that VIP may regulate PRL secretion through two different second messengers, one of which is cyclic AMP and the other as yet unknown. In contrast, the results with ACh (Figs. 6 and 7B) show complete correlations between the effects of ACh on adenylate cyclase and cyclic AMP content and its effect on PRL release, whether in the absence or presence of VIP.

Although GH_3 cells differ from normal pituitary mammotrophs in several respects, the coexistence in these cells of receptors for two neurotransmitters which can regulate PRL secretion supports the idea that PRL release may be controlled by multiple chemical signals. From a theoretical viewpoint, the present findings are important because they indicate that GH_3 cells can be used as a model system to study the interaction between two neuromodulators which affect the transducer component (adenylate cyclase) of receptors which function to modulate PRL secretion.

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