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Regulation of Ca^{2+}-Dependent Cyclic AMP Accumulation and Ca^{2+} Metabolism in Intact Pituitary Tumor Cells by Modulators of Prolactin Production

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SUMMARY

The responsiveness of anterior pituitary tumor (GH_3) cells to promoters of prolactin secretion and/or synthesis and cyclic AMP accumulation was studied as a function of cellular Ca^{2+} content. GH_3 cells exposed to media containing 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid were reduced 7-fold in Ca^{2+} content without loss of viability. Preparations of Ca^{2+}-depleted cells were largely unchanged in cyclic AMP content when challenged by thyrotropin-releasing hormone (TRH), whereas cells which were subsequently restored at optimal Ca^{2+} (0.5 mM) responded to the hormone with 2- to 3-fold increases in cyclic AMP content. The decreased responsiveness of Ca^{2+}-depleted cells to TRH was not influenced by phosphodiesterase inhibitors, incubation time, or hormone concentration. TRH-dependent cyclic AMP accumulation was markedly potentiated by forskolin in Ca^{2+}-restored, but not in Ca^{2+}-depleted, cell preparations. Forskolin extended the time period during which cyclic AMP accumulated in response to TRH without altering the TRH concentration dependency of the cells. Varying increases in GH_3 cyclic AMP content occurred in response to other hormones or agents which enhance prolactin secretion and/or synthesis. In Ca^{2+}-restored cells, cyclic AMP content was increased 2-fold by prostaglandin E_1 (PGE_1) and epidermal growth factor (EGF), 10- to 15-fold by vasoactive intestinal polypeptide (VIP) and 6-fold by phorbol myristate acetate (PMA); the capacity of Ca^{2+}-depleted cells, however, to accumulate cyclic AMP in response to PGE_1, EGF, and VIP was greatly reduced. Accumulation of cyclic AMP following short-term incubations with cholera toxin similarly was dependent on Ca^{2+}. Exposure of GH_3 cells preloaded with ^{45}Ca to TRH, PGE_1, EGF, PMA, or VIP resulted in losses of cell-associated ^{45}Ca. Pretreatment with these agents resulted in a decreased capacity of the cells to accumulate ^{45}Ca from the extracellular medium. The results of this study support the hypothesis that various putative humoral regulators of prolactin secretion and/or synthesis act on GH_3 cells to alter intracellular Ca^{2+} metabolism which in turn results in an increased cyclic AMP content through stimulation of adenylate cyclase activity.

INTRODUCTION

Studies with methylxanthines and cyclic AMP analogues in intact anterior pituitary tissue led to the proposal in 1975 that cyclic AMP regulates prolactin secretion by specialized cells of the anterior pituitary (1). Since regulators of prolactin synthesis and/or secretion such as TRH[1] may affect more than one cell type in the anterior pituitary, well-characterized cloned strains of pituitary tumor (GH) cells are frequently employed to elucidate the mechanism of action of specific regulatory factors. GH cells synthesize and secrete prolactin in response to a variety of humoral substances (2), including TRH and VIP; accumulation of cyclic AMP response to both agents has been observed (2, 3). Analogues of cyclic AMP, phosphodiesterase inhibitors, and cholera toxin (4–6) enhance prolactin synthesis in GH cells and mimic the effects of TRH on prolactin release (4, 5). It has been proposed (5) that cyclic AMP activates a protein kinase in these cells which is subsequently involved with the phosphorylation of proteins required for the secretory process, for gene transcription, or for translation. However, TRH stimulation of thyrotropin or prolactin release

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[1] The abbreviations used are: TRH, thyrotropin-releasing hormone; VIP, vasoactive intestinal polypeptide; Tes, *N*-[tris(hydroxymethyl)-methyl-2-amino]ethanesulfonic acid; EGF, epidermal growth factor; PGE_1, prostaglandin E_1; PMA, phorbol myristate acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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is reported to be dissociated from changes in cyclic AMP concentration; therefore, considerable controversy has arisen regarding the role of cyclic AMP in prolactin secretion and/or synthesis (7).

Evidence that Ca^{2+} stimulates prolactin synthesis and secretion and serves as the primary intracellular mediator of TRH action in GH cells is extensive. Reduction of extracellular Ca^{2+} inhibits prolactin release (8) and decreases total prolactin synthesis several-fold (9). Concentrations of mRNA for the prolactin sequence increase in parallel with Ca^{2+}-induced increases in the rate of prolactin synthesis (10). TRH produces Ca^{2+}-dependent increases in action potential frequency (11–13) and rapid changes in intracellular Ca^{2+} content (14–16). Recently TRH and cyclic AMP were reported to activate distinctive, but overlapping, pathways of protein phosphorylation in GH cells (17, 18), providing evidence against a role for cyclic AMP as the intracellular mediator of TRH action.

Clarification of the interrelationships which exist between Ca^{2+} and cyclic AMP in the anterior pituitary should provide further insight into the mechanisms governing prolactin secretion and/or synthesis. Synthesis of cyclic AMP in intact neural tissue is believed to be regulated by Ca^{2+} and by the intracellular Ca^{2+} receptor protein, calmodulin (19, 20). A calmodulin-dependent form of adenylate cyclase from cerebral cortex has been characterized (21). Although anterior pituitary cells do not originate from cells of the neural crest, homogenates of GH_3 cells possess a Ca^{2+}-stimulated adenylate cyclase activity which, unlike the brain enzyme, does not appear to possess a dissociable calmodulin unit (22). Evidence is lacking, however, that Ca^{2+} regulates cyclic AMP formation in intact, prolactin-producing pituitary cells. The present study was undertaken to assess the role of Ca^{2+} in TRH-dependent cyclic AMP accumulation and to ascertain whether other regulators of prolactin secretion and/or synthesis alter both cyclic AMP and Ca^{2+} metabolism in intact GH cells.

EXPERIMENTAL PROCEDURES

Materials. Medium and sera were purchased from Grand Island Biological Company (Grand Island, N. Y.). Methylisobutylxanthine, Tes, TRH, PGE_1, PMA, 4-α-D-phorbol, VIP, cyclic AMP, 2,3,5-D-triiodothyronine, dopamine hydrochloride, histamine hydrochloride, carbamylcholine chloride, and hydrocortisone 21-hemisuccinate were obtained from Sigma Chemical Company (St. Louis, Mo.). Cholera toxin was purchased from Schwarz/Mann (Orangeburg, N. Y.), forskolin from Calbiochem-Behring (San Diego, Calif.), and receptor-grade EGF from Collaborative Research, Inc. (Waltham, Mass.). [^{125}I]-Labeled 2′-*O*-succinyl cyclic AMP tyrosine methyl ester (>150 Ci/mmole), $^{45}CaCl_2$ (40.5 mCi/mg), and [*methoxy*-^{3}H]inulin (515 Ci/mole) were obtained from New England Nuclear Corporation (Boston, Mass.). Calmodulin was purified from bovine brain as described previously (23).

Cell culture. The GH_3 strain of rat pituitary tumor cells was obtained from the American Type Culture Collection, maintained in monolayer culture in Ham's F-10 medium supplemented with 12.5% horse serum and 5% fetal calf serum and subcultured as described (2). Stock cultures were grown to late logarithmic phase without antibiotics in plastic culture flasks (Falcon, 25 cm^2) at 37°. Experimental cultures were grown at 37° in glass roller bottles, 23 cm in length and 11 cm in diameter, with 400 ml of complete medium containing penicillin (25 units/ml) and streptomycin (50 μg/ml). Cells from three stock flasks were used to initiate growth of experimental cultures which were harvested following 12–14 days of growth.

Cells adapted over a 3-month period to serum-free conditions, in monolayer culture in medium supplemented with transferrin and growth factors as described by Hayashi and Sato (24), were employed to complement the data of Table 1.

Preparation of Ca^{2+}-depleted and Ca^{2+}-restored cells. Cells were detached from glass surfaces with a plastic scraper and the cell suspension was centrifuged at 25° for 5 min at 600 × *g*. Pellets of cells from one roller bottle were suspended in 100 ml of a buffered saline solution containing 137 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1 mM EGTA, and 25 mM Tes (pH 7.5). The cell suspension was centrifuged as described above and the cell pellet was resuspended in another 100 ml of the buffered saline. The cell suspension was then incubated at 37° for 20 min and recentrifuged, and the pellet was resuspended in 100 ml of the buffered saline. Following a final centrifugation the cells were suspended in Tes-buffered saline containing 1 mM EGTA and 1 mM $MgCl_2$. Ca^{2+}-restored cells were prepared by adding $CaCl_2$ to a concentration of 3 mM (2 mM in excess of EGTA) to a portion of the suspension of Ca^{2+}-depleted cells. Unless indicated otherwise, aliquots of Ca^{2+}-depleted and Ca^{2+}-restored cell suspensions in glass tubes were pre-equilibrated at 37° for 30 min. Hormones, drugs, or other additives were dissolved in buffered saline and added to cells in 10- to 20-μl volumes per milliliter of cell suspension. Concentrated stock solutions of PMA or 4-α-D-phorbol were prepared in dimethyl sulfoxide and diluted in saline prior to use. Viability of cell preparations, as assessed by exclusion of trypan blue dye, was routinely 90% ± 5%. The cyclic AMP content of Ca^{2+}-restored cells was unchanged from that of nondepleted cells under a variety of incubation conditions.

Determination of cyclic AMP content. Extracts of cell suspensions were prepared as described previously (25). Cell extracts were acetylated with acetic anhydride and assayed for cyclic AMP by the radioimmunoassay procedure of Harper and Brooker (26), using [^{125}I]-labeled 2′-*O*-succinyl cyclic AMP tyrosine methyl ester as ligand and a rabbit antibody prepared according to the method of Steiner *et al.* (27). Results are expressed as means ± standard deviation of triplicate or quadruplicate incubations from a single preparation of cells in a single experiment. Each experiment was performed at least three times to verify results.

Ca^{2+} flux studies. Measurements of cell-associated ^{45}Ca and ^{45}Ca uptake were performed according to modifications of the procedures of Tan and Tashjian (15). Cells were suspended in growth medium and then washed

twice by centrifugation and resuspension in fresh Tes-buffered Ham's F-10 medium (pH 7.4) containing 1% serum albumin at room temperature. For measurements of cell-associated Ca^{2+}, cell suspensions were incubated at 37° for 4 hr with $^{45}CaCl_2$ (2 μCi/ml) (specific activity, 1.5 μCi/μmole). As reported previously for GH_4C_1 cells in monolayer culture (15), cell ^{45}Ca content reached a maximum at 2 hr of incubation and remained constant during the next 2 hr of incubation. Agents to be examined for effects on cell Ca^{2+} content were then added and the incubation was continued for an additional 15 min. Aliquots of cell suspension (0.5 ml) were withdrawn and added to 10 ml of ice-cold 150 mM NaCl containing 2.5 mM $LaCl_3$ and 1.5% (w/v) serum albumin in conical glass centrifuge tubes. Following centrifugation at 1000 × *g* for 5 min at 4°, supernatant fluids were removed and the tubes were inverted to drain. Traces of solution were removed from the sides of the tubes with cotton swabs, and the cell pellets were dissolved in saline containing 1% Triton X-100. Aliquots of dissolved cells were then analyzed for radioactivity. Unlabeled cells and ^{45}Ca were added separately to the saline-$LaCl_3$ solution described above to assess the contribution of extracellular ^{45}Ca; values obtained constituted the blanks and were subtracted from experimental values. Blank values were routinely 5%–10% of experimental values. Results are expressed as the average ± range of values (five or six per test substance) obtained.

For measurements of ^{45}Ca uptake, cells suspended in fresh, buffered Ham's F-10 medium were pretreated with test substances for 30 min at 37°. ^{45}Ca was then added and the incubation was continued for an additional 4 hr. Aliquots of cell suspension (0.5 ml) were analyzed for cellular ^{45}Ca by the centrifugation procedure described above.

The capacity of Ca^{2+}-depleted cells to take up ^{45}Ca following a pretreatment with various agents was also examined. Ca^{2+}-depleted cells were prepared as described above and were pre-equilibrated for 30 min at 37° in Tes-buffered saline containing 1 mM $MgCl_2$, 1 mM EGTA, and 60 μM Na_2HPO_4. Cells were then incubated for 5 min with test substances and subsequently with 1.5 mM ^{45}Ca (4 $\mu Ci \cdot \mu mole^{-1}$) for an additional 5 min. Aliquots of cell suspension were removed and cellular ^{45}Ca was determined as described above.

Cyclic AMP phosphodiesterase activity. Cells were washed with the Tes-buffered saline solution described above and were harvested by centrifugation at 600 × *g* for 5 min and suspended in 10 mM imidazole (pH 7.5) containing 3 mM $MgCl_2$. Suspensions were homogenized by sonic disruption and analyzed for cyclic nucleotide phosphodiesterase under standard conditions (28) at 0.1 mM EGTA with cyclic AMP as substrate. Ca^{2+}, calmodulin, and cyclic AMP concentrations were as indicated in the text. Values were corrected for controls without enzyme.

Miscellaneous. Ca^{2+} contents of standard solutions of cells and of their extracellular fluids were measured with a Model 303 Perkin-Elmer atomic absorption spectrophotometer equipped with a Model 2200 HGA graphite furnace. At least five determinations were made for each sample. The standard error of the mean, based on three different cell preparations, was ±10%. Protein concentrations were determined by the method of Sedmak and Grossberg (29), using bovine serum albumin as standard.

TABLE 1

Cyclic AMP content and cyclic AMP phosphodiesterase activities of GH_3 cells grown in the presence of different fetal calf serum preparations

GH_3 cells obtained from the same seed stock preparation were grown to late logarithmic phase in culture flasks in complete growth medium with the fetal calf serum lot number indicated or under serum-free conditions. A, Ca^{2+}-depleted and Ca^{2+}-restored cells were prepared from each set of cultures and were assayed for cyclic AMP content following 1 min of incubation with 0.1 μM TRH or saline. B, Sonically disrupted homogenates of cells grown with different fetal calf serum preparations were analyzed for cyclic nucleotide phosphodiesterase at the indicated cyclic AMP concentrations. EGTA (0.1 mM) was present in all incubations. Ca^{2+} and calmodulin, where added, were 0.2 mM and 1 μg, respectively. Activities are expressed as total cyclic nucleotide phosphodiesterase present in incubations under the conditions indicated.

A. Cyclic AMP content

Serum Lot no.	Ca^{2+} restoration of cells	−TRH	+TRH
		pmoles·mg protein^{-1}	
31K2913	−	1.5 ± 0.2	1.9 ± 0.1
31K2913	+	1.6 ± 0.3	2.9 ± 0.3
32P9121	−	2.9 ± 0.2	3.3 ± 0.1
32P9121	+	1.8 ± 0.1	3.5 ± 0.2
None (serum-free)	−	1.6 ± 0.2	1.9 ± 0.1
None (serum-free)	+	1.5 ± 0.2	3.0 ± 0.3

B. Cyclic AMP phosphodiesterase activity

Serum Lot no.	Assay conditions	Cyclic AMP concentration		
		0.25 μM	2.5 μM	25 μM
		pmoles cyclic AMP hydrolyzed·min^{-1}·mg protein^{-1}		
31K2913	EGTA	33	220	1230
31K2913	Ca^{2+} + calmodulin	83	450	2260
32P9121	EGTA	24	120	400
32P9121	Ca^{2+} + calmodulin	38	240	740

RESULTS

Ca^{2+} dependence of cyclic AMP accumulation in response to TRH. The effect of Ca^{2+} on the cyclic AMP content of unstimulated GH_3 cells was observed initially to vary with the lot of fetal calf serum in which the culture had been grown (Table 1A). Serum lots fell routinely into two categories, as typified by lot numbers 31K2913 and 32P9121, with respect to the cyclic AMP content of unstimulated Ca^{2+}-depleted cells. Cells harvested from some sera (e.g., Lot 31K2913) upon subsequent Ca^{2+} depletion possessed cyclic AMP contents either slightly lower than or equal to that of their Ca^{2+}-restored counterparts, whereas cells from other sera (e.g., Lot 32P9121), when Ca^{2+}-depleted, exhibited significantly higher cyclic AMP contents than when Ca^{2+}-restored. The cyclic AMP content of Ca^{2+}-restored cells, however, was largely invariant with fetal calf serum lot. TRH produced an accumulation of cyclic AMP in Ca^{2+}-restored cells grown with either serum but was significantly less effective in raising the cyclic AMP content of Ca^{2+}-depleted cells regardless of their initial nucleotide

content. Cells grown serum-free exhibited cyclic AMP contents and TRH responses which were similar to those of cells grown with Lot 31K2913.

The observed variability in the cyclic AMP content of unstimulated Ca^{2+}-depleted cells harvested from different growth sera appeared to correlate with variances found in cellular cyclic AMP phosphodiesterase activities (Table 1B). Analyses of cyclic AMP phosphodiesterase activities from cell homogenates indicated that cells produced from serum Lot 31K2913 possessed 2- to 3-fold more phosphodiesterase activity than did cells from Lot 32P9121, with proportionate elevations being found for both the Ca^{2+}-dependent and Ca^{2+}-independent forms of the activity. Cells harvested from Lot 31K2913 when Ca^{2+}-depleted had low basal cyclic AMP content which correlated with a high Ca^{2+}-independent phosphodiesterase activity. By contrast, cells from Lot 32P9121 (which are low in phosphodiesterase activity) when Ca^{2+}-depleted had a relatively high cyclic AMP content which was lowered to the basal value by Ca^{2+} restoration, presumably as a reflection of the activation of the Ca^{2+}-dependent phosphodiesterase activity. Similar correlations appear to hold for the cyclic AMP content of Ca^{2+}-depleted TRH-stimulated cells. The cyclic AMP content of TRH-stimulated Ca^{2+}-restored cells, however, appeared to reflect a balance between Ca^{2+} stimulation of both synthesis and degradation. For the purpose of consistency, experiments to follow were performed with cells grown in the presence of fetal calf serum Lot 31K2913.

To verify that cells prepared with solutions containing EGTA were Ca^{2+}-depleted, the Ca^{2+} content of cells in EGTA-containing medium was compared with that of cells in medium containing 2 mM Ca^{2+} in excess of EGTA. Cells were separated from their extracellular medium by centrifugation and were analyzed for Ca^{2+} content by atomic absorption spectrophotometry (Table 2A). The Ca^{2+} content of Ca^{2+}-depleted cells was found to be 14% of that present in Ca^{2+}-restored preparations. It was presumed that extracellular bound Ca^{2+} did not contribute significantly to the value obtained for Ca^{2+}-restored cells since a Ca^{2+} determination of the latter following centrifugation through a $LaCl_3$-containing saline solution provided a similar value. The effect of a phosphodiesterase inhibitor on the cyclic AMP content of Ca^{2+}-depleted and Ca^{2+}-restored cells and on the responses of these cell preparations to TRH was also examined (Table 2B). Methylisobutylxanthine (1 mM) increased the cyclic AMP content of unstimulated cells approximately 40-fold; the nucleotide content of Ca^{2+}-restored cells was slightly lower than that of Ca^{2+}-depleted cells in the presence of inhibitor. As was observed without inhibitor, TRH increased the cyclic AMP content of Ca^{2+}-restored cells 2-fold; no cyclic AMP accumulation was observed, however, in Ca^{2+}-depleted cells.

The time dependence of TRH-stimulated cyclic AMP accumulation in Ca^{2+}-depleted and Ca^{2+}-restored cells is shown in Fig. 1. A rapid increase in cyclic AMP content was apparent in Ca^{2+}-restored cells; maximal responses (2.5-fold increases over basal) were observed within 0.5–2 min following addition of hormone. After 2 min the cyclic AMP content was observed to decline, and basal levels of nucleotide were reattained approximately 30 min after TRH addition. Ca^{2+}-depleted cells treated with TRH exhibited smaller increases in cyclic AMP content; the greatest increase over basal cyclic AMP content (40% stimulation) was observed within 0.5 min following TRH addition.

TABLE 2

Ca^{2+} and cyclic AMP content of Ca^{2+}-depleted and Ca^{2+}-restored GH_3 cells

A. Ca^{2+}-depleted or Ca^{2+}-restored cells were pretreated for 45 min in new polypropylene tubes. Cells were separated from their extracellular media by centrifugation at 600 × *g* for 5 min. After allowing the tubes to drain, the insides were wiped dry with cotton swabs and the pellets of cells were suspended in Ca^{2+}-free water. To remove extracellular bound Ca^{2+} from Ca^{2+}-restored preparations, aliquots of the latter were centrifuged through 10 ml of ice-cold saline containing 2.5 mM $LaCl_3$ and 1% serum albumin, and samples prepared as above. All cell samples were then analyzed for Ca^{2+} content by atomic absorption spectrophotometry. Contributions of extracellular Ca^{2+} were estimated from the [^{3}H]methoxyinulin space of each pellet and were subtracted. Values provided represent averages ± range of five determinations per sample. The Ca^{2+} content of the extracellular medium was also determined by atomic absorption spectrophotometry and was found to be 2.7 mM for Ca^{2+}-restored cells and 6.7 μM for Ca^{2+}-depleted cells.

B. Ca^{2+}-depleted and Ca^{2+}-restored cells were pretreated for 30 min with or without 1 mM methylisobutylxanthine and for an additional 2 min with or without 0.1 μM TRH. Cell samples were then analyzed for cyclic AMP content.

A. Determination of Ca^{2+} content

Cells	Ca^{2+} content
	nmoles·mg protein^{-1}
Ca^{2+}-depleted	2.8 ± 0.2
Ca^{2+}-restored	22 ± 2
Ca^{2+}-restored, $LaCl_3$-saline washed	19 ± 1

B. Determination of cyclic AMP content

Cells	Cyclic AMP			
	−Methylisobutylxanthine		+Methylisobutylxanthine	
	−TRH	+TRH	−TRH	+TRH
	pmoles·mg protein^{-1}			
Ca^{2+}-depleted	1.5 ± 0.1	1.8 ± 0.1	90 ± 3	88 ± 3
Ca^{2+}-restored	2.3 ± 0.1	4.5 ± 0.1	70 ± 2	140 ± 4

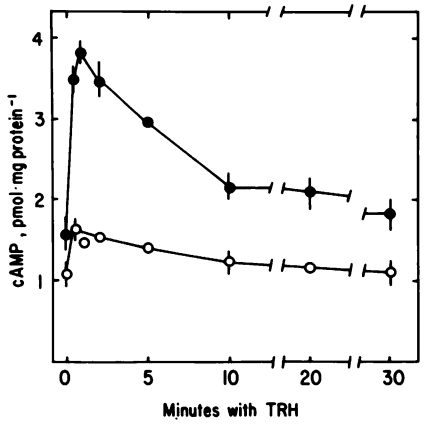

FIG. 1. *Accumulation of cyclic AMP with time in suspensions of Ca^{2+}-depleted and Ca^{2+}-restored GH_3 cells following addition of TRH*

Ca^{2+}-depleted (○) or Ca^{2+}-restored (●) cells were challenged with 0.1 μM TRH. At the indicated times aliquots of cell suspension were removed for cyclic AMP measurements.

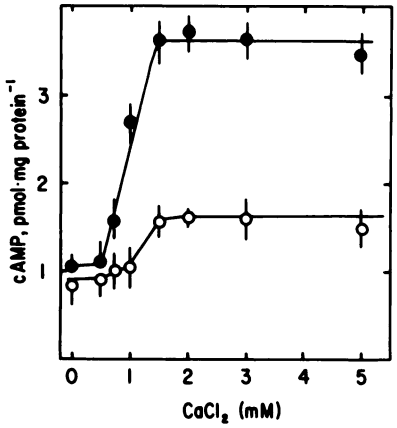

FIG. 2. *Extracellular Ca^{2+} concentration dependence of the cyclic AMP content of GH_3 cells in the absence or presence of TRH*

Ca^{2+}-depleted cells were pretreated for 30 min in extracellular medium containing the indicated Ca^{2+} concentrations. Cells were then incubated for 1 min without (○) or with (●) 0.1 μM TRH, and the cyclic AMP content was determined.

The extracellular $CaCl_2$ concentration dependence of the cyclic AMP content of unstimulated cells and of cells exposed to TRH for 1 min is shown in Fig. 2. Cyclic AMP in unstimulated cells increased slightly as a function of external Ca^{2+} concentration; 1.5 mM added Ca^{2+} increased the cyclic AMP content approximately 50%. Cyclic AMP accumulated in response to TRH was dependent on extracellular Ca^{2+}, with a maximal response (3-fold increase in cyclic AMP content) observed at 1.5 mM added Ca^{2+}. Since the EGTA concentration of the external medium was 1 mM, a free Ca^{2+} concentration approximating 0.5 mM provided maximal TRH responses.

The TRH concentration dependence of cyclic AMP accumulation in Ca^{2+}-depleted and Ca^{2+}-restored cells was also examined (Fig. 3). In Ca^{2+}-restored cells, maximal and half-maximal accumulations of cyclic AMP were obtained with 10 and 3 nM TRH, respectively. The hormone concentration dependence was similar in Ca^{2+}-depleted cells, but increases in cyclic AMP content were considerably smaller at all TRH concentrations tested.

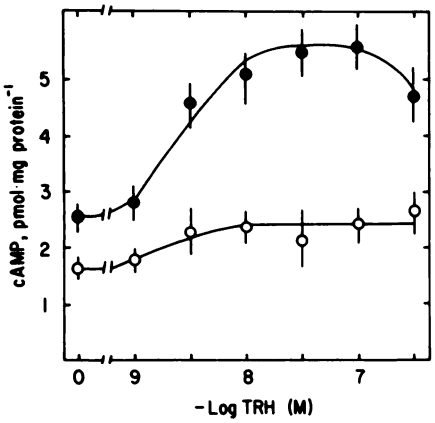

FIG. 3. *TRH concentration dependence of cyclic AMP accumulation in Ca^{2+}-depleted and Ca^{2+}-restored GH_3 cells*

Ca^{2+}-depleted (○) or Ca^{2+}-restored (●) cells were incubated for 1 min with the indicated concentrations of TRH, and the cyclic AMP content was determined.

Potentiation of TRH-dependent cyclic AMP accumulation by forskolin. The diterpene forskolin has been reported to activate adenylate cyclase in membranes from a variety of tissues via direct interaction with the catalytic subunit (30). In addition, in intact cells forskolin augments receptor-mediated increases in cyclic AMP (30, 31). It was of interest, therefore, to determine whether this agent would augment the increase in cyclic AMP seen in GH_3 cells following TRH. As shown in Table 3, the cyclic AMP content of Ca^{2+}-depleted and Ca^{2+}-restored GH_3 cells was enhanced 10-fold by 1 μM forskolin and 70-fold by 10 μM drug. When Ca^{2+}-depleted cells were incubated with TRH in combination with forskolin, the cyclic AMP content was equivalent to that obtained with forskolin alone. In Ca^{2+}-restored cells, however, a combination of TRH and 1 or 10 μM forskolin resulted in a marked potentiation of cyclic AMP accumulation over that seen with TRH or forskolin alone.

The influence of forskolin on the time dependence of TRH-stimulated cyclic AMP accumulation in cells suspended in Ca^{2+}-containing medium (nondepleted cells) was then examined (Fig. 4A). TRH alone produced the characteristic rapid rise and fall in cyclic AMP concentration. Forskolin (0.5 μM) alone produced a rapid increase in cyclic AMP; 2 min of incubation with the drug produced an 8-fold increase in nucleotide content which was maintained throughout the remainder of the 30-min incubation period. TRH in combination with forskolin produced synergistic elevations of cyclic AMP at all incubation times. Maximal cyclic AMP content (a 3-fold increase over that of forskolin alone) was maintained for at least 6 min of incubation. After 8 min of incubation with both agents the cyclic AMP content was observed to decline slowly.

Daly *et al.* (31) observed in brain slices that augmentation by forskolin of cyclic AMP accumulation in response to a variety of hormones involves either an enhanced efficacy or an increased apparent potency for a given hormone. The potentiation by forskolin of TRH-dependent cyclic AMP accumulation in GH_3 cells in the presence of Ca^{2+} was found to involve an enhancement of TRH efficacy (Fig. 4B). Forskolin (0.5 μM) produced a 10- to 15-fold enhancement of hormone-specific cyclic AMP accumulation at all TRH concentrations examined. However, the TRH concentration producing a maximal

TABLE 3

Forskolin concentration dependence of cyclic AMP accumulation in Ca^{2+}-depleted and Ca^{2+}-restored GH_3 cells in the presence and absence of TRH

Ca^{2+}-depleted and Ca^{2+}-restored cells were incubated for 2 min with the indicated concentrations of forskolin and with or without 0.1 μM TRH, and were subsequently analyzed for cyclic AMP content.

Forskolin	Cyclic AMP content			
	−TRH		+TRH	
	Ca^{2+}-depleted cells	Ca^{2+}-restored cells	Ca^{2+}-depleted cells	Ca^{2+}-restored cells
μM	*pmoles·mg protein⁻¹*			
0	1.6 ± 0.3	1.8 ± 0.2	1.9 ± 0.1	3.3 ± 0.2
1	15 ± 2	22 ± 2	18 ± 2	56 ± 4
10	120 ± 5	130 ± 5	120 ± 5	235 ± 10

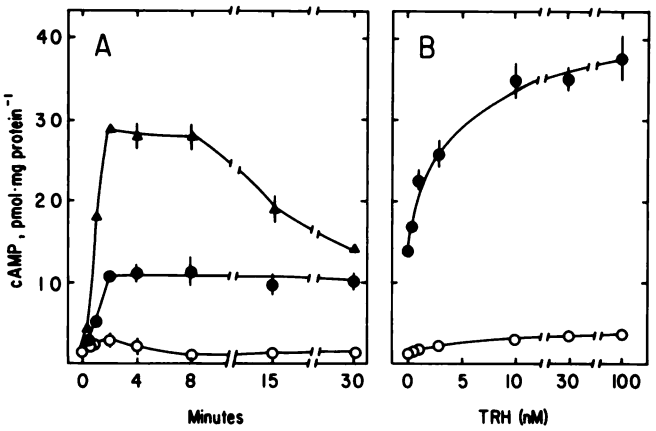

FIG. 4. *Potentiation by forskolin of TRH-dependent cyclic AMP accumulation in GH_3 cells in the presence of Ca^{2+}*

A. Time course of cyclic AMP accumulation. GH_3 cells suspended in medium containing 1 mM $CaCl_2$ and no EGTA were incubated for the indicated times with 0.1 μM TRH (○), with 0.5 μM forskolin (●), or with 0.1 μM TRH and 0.5 μM forskolin (▲), and the cyclic AMP content of the samples was determined.

B. TRH concentration dependence of cyclic AMP accumulation. Cells suspended in medium containing 1 mM $CaCl_2$ and no EGTA were incubated for 2 min with the indicated concentrations of TRH and in the absence (○) or presence (●) of 0.5 μM forskolin, and the cyclic AMP content was subsequently determined.

response (approximately 10 nM) was unchanged in the presence of forskolin.

Cyclic AMP accumulation in response to other modulators of prolactin synthesis and/or secretion. Prostaglandins (32), EGF (33, 34), VIP (3), and the tumor-promoting phorbol ester PMA (35) have been demonstrated to enhance prolactin synthesis and/or secretion in GH cells. In view of the proposals that Ca^{2+} and cyclic AMP modulate prolactin synthesis or secretion or both, it was of interest to test the capacity of such agents to promote cyclic AMP accumulation in a Ca^{2+}-dependent manner. The cyclic AMP contents of Ca^{2+}-depleted and Ca^{2+}-restored GH_3 cells following short-term incubations with a variety of agents in the absence or presence of a phosphodiesterase inhibitor are given in Table 4. Increases in cyclic AMP were observed following treatments with TRH, PGE_1, PMA, EGF, and VIP, with the latter producing the greatest response; cyclic AMP accumulation following TRH, PGE_1, EGF, and VIP was significantly greater in Ca^{2+}-restored than in Ca^{2+}-depleted cell preparations. Histamine, carbamylcholine, the inactive phorbol ester analogue 4-α-D-phorbol, and dopamine, which have not to our knowledge been reported to promote prolactin synthesis and/or secretion in GH_3 cells, did not elevate cyclic AMP concentrations in either Ca^{2+}-depleted or Ca^{2+}-restored cells.

The time dependence and degree of cyclic AMP accumulation varied with the individual agent examined (Fig. 5). Concentrations of agents which produced maximal degrees of cyclic AMP accumulation at 1 min of incubation (data not shown) were chosen for the purpose of comparison. Time courses of and degrees of cyclic AMP accumulation in Ca^{2+}-restored cells following 30 nM PGE_1 and 30 nM EGF were similar to such parameters observed following the addition of 0.1 μM TRH (Fig. 1). In contrast,

TABLE 4

Cyclic AMP contents of Ca^{2+}-depleted and Ca^{2+}-restored GH_3 cells treated with various hormones

Ca^{2+}-depleted and Ca^{2+}-restored cells were pretreated for 20 min without (Experiment I) or with (Experiment II) 1 mM methylisobutylxanthine. Cell preparations were then challenged with agents indicated and analyzed for cyclic AMP content after 2 min of incubation. ND, Not determined.

Agent	Cyclic AMP content			
	Expt I (−methylisobutylxanthine)		Expt II (+methylisobutylxanthine)	
	Ca^{2+}-depleted cells	Ca^{2+}-restored cells	Ca^{2+}-depleted cells	Ca^{2+}-restored cells
	pmoles·mg protein^{-1}			
None	1.4 ± 0.2	2.2 ± 0.2	125 ± 5	70 ± 3
TRH (0.1 μM)	1.8 ± 0.2	4.8 ± 0.1	140 ± 10	130 ± 5
PGE_1 (30 nM)	1.9 ± 0.2	4.5 ± 0.1	125 ± 5	120 ± 10
PMA (0.1 μM)	4.8 ± 0.1	4.4 ± 0.3	230 ± 5	190 ± 5
EGF (50 nM)	1.8 ± 0.4	4.3 ± 0.1	125 ± 5	110 ± 5
VIP (0.1 μM)	9.2 ± 1.0	25 ± 3	470 ± 20	780 ± 40
Histamine (1.0 μM)	1.4 ± 0.2	2.1 ± 0.2	ND	ND
Carbamylcholine (1.0 μM)	1.2 ± 0.2	1.8 ± 0.2	ND	ND
Dopamine (1.0 μM)	1.6 ± 0.2	2.1 ± 0.2	120 ± 5	73 ± 3
4-α-D-Phorbol (0.1 μM)	1.7 ± 0.2	2.5 ± 0.2	125 ± 5	75 ± 3

a 15-fold increase in the cyclic AMP content of Ca^{2+}-restored cells was observed within 1 min of incubation with 0.1 μM VIP; thereafter cyclic AMP concentrations declined rapidly. However, Ca^{2+} dependencies of the responses to VIP as well as to PGE_1 and EGF were apparent at all times following hormone addition. Accumulation of cyclic AMP following PMA differed in two respects from that following hormones. First, cyclic AMP was elevated for longer time periods; the maximal cyclic AMP content (a 4- to 6-fold elevation of cyclic AMP over basal level) was attained at 3 min following addition of

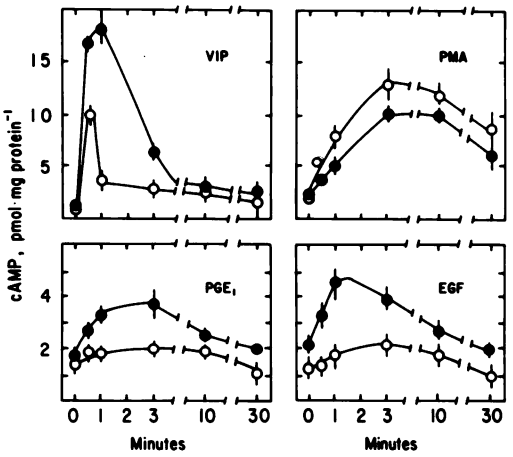

FIG. 5. *Accumulation of cyclic AMP with time in Ca^{2+}-depleted and Ca^{2+}-restored GH_3 cells treated with various agents*

Ca^{2+}-depleted (○) and Ca^{2+}-restored (●) cells were challenged with 0.1 μM VIP (*upper left*), with 0.1 μM PMA (*upper right*), with 30 nM PGE_1 (*lower left*), or with 30 nM EGF (*lower right*). At the indicated time periods following addition of the agent, samples were withdrawn for cyclic AMP measurements.

drug and was maintained for another 7 min of incubation. Whereas cyclic AMP responses to TRH, VIP, PGE_1, or EGF were attenuated at 30 min, the cyclic AMP content of cells treated for a similar time period with PMA was elevated 2.5- to 3-fold. Second, cyclic AMP accumulation in response to PMA was not enhanced by Ca^{2+}. In fact, the cyclic AMP content of Ca^{2+}-depleted cells was somewhat greater than that of Ca^{2+}-restored cells at all times following addition of drug.

Cholera toxin, which irreversibly activates adenylate cyclase by catalyzing an ADP-ribosylation of the guanyl nucleotide regulatory component of the enzyme (36–39), enhances prolactin synthesis and release by GH_3 cells (6). Since cyclic AMP accumulation and activation of adenylate cyclase following treatment of glial tumor cells with cholera toxin is dependent on intracellular Ca^{2+} (25), it was of interest to assess the role of Ca^{2+} in cholera toxin-stimulated cyclic AMP accumulation in GH_3 cells. Ca^{2+}-depleted and Ca^{2+}-restored cells were treated with 2 nM cholera toxin; at varying times of incubation the cells were harvested by centrifugation and the cyclic AMP content was determined (Fig. 6). Twenty minutes of incubation were required to detect an increase in the cyclic AMP content of Ca^{2+}-restored cells. Thereafter the cyclic AMP content increased rapidly, and maximal accumulation of nucleotide was obtained 60 min after toxin addition. In Ca^{2+}-depleted cells, however, cyclic AMP was accumulated more slowly, with detectable increases in nucleotide observable after 30 min of incubation with toxin. More than 90 min of incubation with toxin were required for maximal cyclic AMP (90 pmol·mg protein^{-1}) to be attained in Ca^{2+}-depleted cells.

^{45}Ca flux studies. Several recent studies (14–16) have provided evidence that TRH promotes an efflux of Ca^{2+} from GH cells. Other modulators of prolactin synthesis and/or secretion, however, have not been reported to regulate Ca^{2+} metabolism in these cells. In view of the Ca^{2+} dependence of cyclic AMP accumulation in response to several prolactin-modulating agents, it was of interest to examine the effects of such agents on GH_3 cell Ca^{2+} content. Initially, cells preloaded with ^{45}Ca for 4 hr in Ham's medium were incubated for 15 min with test agents, and cell-associated ^{45}Ca was measured (Table 5). A 50% loss of cell-associated ^{45}Ca was observed following treatment with TRH and VIP. Treatment with PMA, PGE_1, and EGF resulted in 32%, 25%, and 18% losses, respectively, in cell-associated ^{45}Ca. Methylisobutylxanthine, however, did not affect cell ^{45}Ca content.

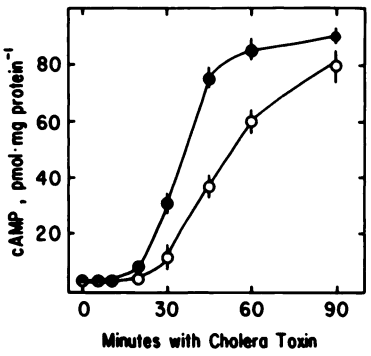

FIG. 6. *Accumulation of cyclic AMP with time in Ca^{2+}-depleted and Ca^{2+}-restored GH_3 cells following addition of cholera toxin*

Ca^{2+}-depleted (○) or Ca^{2+}-restored (●) cells were challenged with 1 nM cholera toxin. Aliquots of cell suspension (1 ml) were removed at the indicated times following toxin addition and centrifuged immediately at 1000 × *g* for 2 min at 4°. Extracellular fluids were discarded, and cell pellets were analyzed for cyclic AMP content.

TABLE 5

Effects of various agents on total ^{45}Ca content of GH_3 cells preloaded with ^{45}Ca

Washed cells were suspended in Tes-buffered Ham's F-10 medium (pH 7.4) containing 1% serum albumin. Suspensions of cells were incubated at 37° for 4 hr with ^{45}Ca (2 μCi/ml) (1.5 μCi·μmole^{-1}). The agents indicated were then added to cell suspensions, and the incubation period was continued for an additional 15 min. Aliquots of cell suspensions (five per experimental value) were withdrawn, and measurements of cell-associated ^{45}Ca were performed.

Agent	Cell-associated ^{45}Ca
	cpm $^{45}Ca \cdot 10^{-3} \cdot$ mg protein^{-1}
None	19.4 ± 1.9
TRH (0.1 μM)	10.9 ± 1.7
PMA (0.1 μM)	13.2 ± 1.2
PGE_1 (30 nM)	14.6 ± 0.3
EGF (30 nM)	15.9 ± 1.0
VIP (0.1 μM)	9.5 ± 1.5
Methylisobutylxanthine (1 mM)	21.3 ± 2.5

The capacity of GH_3 cells which had been pretreated with various agents to take up ^{45}Ca was then examined. Cells were pretreated for 30 min in Ham's medium with test agents and then incubated an additional 4 hr with ^{45}Ca. As shown in Table 6, calcium uptake was reduced 20% by TRH and PGE_1, 53% by PMA, and 27% by VIP. In contrast, hydrocortisone, cholera toxin, triiodothyronine, methylisobutylxanthine, and 4-α-D-phorbol had no effect on calcium uptake. Ca^{2+}-depleted GH_3 cells, pretreated for 5 min with various agents, were also tested for their capacity to accumulate ^{45}Ca during a 5-min incubation with the cation (Table 7). ^{45}Ca uptake by Ca^{2+}-depleted cells was routinely inhibited 40%–80% by TRH, PGE_1, PMA, EGF, and VIP. On the other hand, pretreatment with 4-α-D-phorbol, cholera toxin, or meth-

TABLE 6

^{45}Ca uptake by GH_3 cells pretreated with various agents

Washed cells were suspended in Tes-buffered Ham's F-10 medium (pH 7.4) containing 1% serum albumin and 1.3 mM $CaCl_2$, and aliquots of cell suspension were pretreated for 30 min with the agents indicated. ^{45}Ca (2 μCi/ml) (1.5 μCi·μmole^{-1}) was then added and cell suspensions were incubated for 4 hr. Samples of treated cells suspensions (five per experimental value) were withdrawn for determinations of cell ^{45}Ca content.

Agent	Calcium uptake
	nmoles·mg protein^{-1}
None	7.7 ± 0.1
TRH (0.1 μM)	6.1 ± 0.2
PGE_1 (30 nM)	6.2 ± 0.2
PMA (0.1 μM)	3.6 ± 0.2
VIP (0.1 μM)	5.6 ± 0.2
Hydrocortisone (0.1 μM)	7.4 ± 0.3
Cholera toxin (2.4 nM)	8.0 ± 0.3
Triiodothyronine (2 nM)	7.5 ± 0.1
Methylisobutylxanthine (1 mM)	7.5 ± 0.3
4-α-D-Phorbol (0.1 μM)	7.6 ± 0.2

TABLE 7

^{45}Ca uptake by Ca^{2+}-depleted GH_3 cells pretreated with various agents

Ca^{2+}-depleted cells in buffered saline containing 1 mM EGTA were pretreated for 5 min with the agents indicated. Pretreated cells were then incubated for 5 min with 1.5 mM ^{45}Ca (4 $\mu Ci \cdot \mu mole^{-1}$), and aliquots of cell suspension (six per experimental value) were withdrawn for measurements of ^{45}Ca uptake. I, II, and III represent three separate experiments. ND, Not determined.

Agent	Calcium uptake		
	I	II	III
	nmoles·mg protein^{-1}		
None	1.4 ± 0.2	1.1 ± 0.2	1.2 ± 0.2
TRH (0.1 μM)	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.2
PGE_1 (30 nM)	0.3 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
PMA (0.1 μM)	0.4 ± 0.1	0.3 ± 1	ND
4-α-D-Phorbol (0.1 μM)	1.2 ± 0.3	ND	ND
EGF (30 nM)	0.5 ± 0.1	ND	0.6 ± 0.1
VIP (0.1 μM)	0.3 ± 0.1	ND	0.4 ± 0.2
Cholera toxin (2.4 nM)	ND	1.0 ± 0.1	ND
Methylisobutylxanthine (1 mM)	ND	1.1 ± 0.1	ND

ylisobutylxanthine did not influence ^{45}Ca uptake by Ca^{2+}-depleted cells.

DISCUSSION

The results presented in this report demonstrate that depletion of cellular Ca^{2+} reduces the capacity of GH_3 cells to accumulate cyclic AMP in response to TRH, EGF, PGE_1, and VIP and retards the effect of cholera toxin on cyclic AMP accumulation in these cells. Restoration of the extracellular medium with Ca^{2+} at concentrations believed to prevail in extracellular fluids (i.e., 0.5–1 mM free cation) restored maximal cyclic AMP accumulation in response to TRH (Fig. 2) and to other agents.[2] A number of observations support the conclusion that enhancement by Ca^{2+} of cyclic AMP accumulation in GH_3 cells reflects the activation of a Ca^{2+}-dependent form of adenylate cyclase. First, forskolin, which magnifies the stimulatory effects of hormones and GTP analogues on adenylate cyclase activity by activating the catalytic unit of the enzyme (30, 31), potentiated TRH-dependent cyclic AMP accumulation only in Ca^{2+}-restored cells (Table 3). Second, Ca^{2+} enhanced cyclic AMP accumulation in response to cholera toxin (Fig. 6), a substance which has been established to exert its effects via an irreversible activation of adenylate cyclase. Third, the adenylate cyclase activity of cell-free preparations of GH_3 cells was previously shown to be stimulated by micromolar free Ca^{2+} concentrations (22). Last, it is improbable that Ca^{2+} increases cyclic AMP content by inhibiting a cyclic AMP phosphodiesterase, since high concentrations of a potent phosphodiesterase inhibitor served to enhance (Table 4) and prolong[2] cyclic AMP accumulation in response to TRH, VIP, PMA, PGE_1, and EGF in Ca^{2+}-restored, but not in Ca^{2+}-depleted cells. In addition, Ca^{2+} stimulated total cyclic AMP phosphodiesterase activity of cell-free preparations of GH_3 cells (Table 1). Ca^{2+}-dependent phosphodiesterase may, however, play a role in the rapid attenuation of cyclic AMP responses to the forementioned agents.

The Ca^{2+} requirement for cyclic AMP accumulation in GH_3 cells appears to be regulatory rather than constitutive. Concomitant with increases in cyclic AMP content, changes in cell Ca^{2+} content were observed following treatment with TRH, EGF, PGE_1, VIP, and PMA. Both cell-associated ^{45}Ca (Table 5) and ^{45}Ca uptake (Tables 6 and 7) were reduced in accord with a promotion by these agents of mobilization of Ca^{2+} from intracellular to extracellular pools. It is probable that those agents release Ca^{2+} from sequestration at intracellular storage sites to intracellular free Ca^{2+} pools (7). The immediate effects of such mobilization of Ca^{2+} appear to include (a) an enhancement of cyclic AMP synthesis and (b) the activation of a Ca^{2+}-dependent ATPase such as has been described in erythrocytes and which is believed responsible for the transport of Ca^{2+} across the plasmalemma into extracellular fluids (40).

These data are also consistent with the notion that cyclic AMP accumulation in GH_3 cells is dependent on a rapidly exchangeable, membrane-associated Ca^{2+} pool, perhaps related to the "superficial" pool proposed by Tan and Tashjian (15) to exist in GH_4C_1 cells. One Ca^{2+} pool of the GH_4C_1 cells was described as residing in a superficial compartment in that it was rapidly exchangeable ($t_{1/2} \simeq 1$ min) and removable by proteinase treatment. The other pool was believed to have a slower exchange rate ($t_{1/2} \simeq 28$ min) and was not affected by proteinase treatment. TRH was reported to stimulate both an acute release of ^{45}Ca from the superficial compartment into the medium and a slower ^{45}Ca uptake into the intracellular compartment; the increased uptake was believed to involve an accelerated rate of Ca^{2+} exchange between the extracellular medium and the intracellular compartment and a small increase in the size of the intracellular exchangeable Ca^{2+} pool. The Ca^{2+} pool in GH_3 cells which enhances cyclic AMP accumulation following TRH, PMA, PGE_1, EGF, or VIP is likely to be associated with the cell plasmalemma or a superficial compartment for several reasons. The components of adenylate cyclase and receptors for VIP, EGF, and PGE_1 are recognized to be associated with the cell membrane, and PMA expresses a variety of actions at the cell surface (41). In addition, the times of onset of cyclic AMP accumulation in GH_3 cells in response to TRH (Fig. 1) and of Ca^{2+} exchange within the superficial pool of GH_4C_1 cells following TRH (15) are similar.

The rapid attenuation of cyclic AMP responses to TRH and to other hormones may explain why some workers do not detect alterations in cyclic AMP content following treatment of organ cultures of rat pituitary or thyrotroph-enriched cell preparations with TRH (42). Low concentrations of forskolin appear to be capable of revealing a receptor-mediated activation of cyclic AMP that normally might not be detected (30, 31). The studies reported here with forskolin leave little doubt that TRH increases the cyclic AMP content of GH_3 cells. While TRH alone increased cyclic AMP content 2- to 3-fold at 1–2 min of incubation, TRH in the presence of forskolin enhanced cyclic AMP content 3-fold over that of forskolin alone for at least 10 min of incubation (Fig. 4A). The

[2] M. A. Brostrom, unpublished observation.

increment in cyclic AMP accumulation due to TRH was increased 10- to 15-fold by the drug, implying that TRH directly or indirectly affects the catalytic unit of adenylate cyclase. The receptor for TRH is not believed to be coupled to adenylate cyclase because the enzyme in cell-free preparations of pituicytes (42) or GH_3 cells (22, 43) is not stimulated significantly by TRH. Alternately, as proposed above, the hormone may affect adenylate cyclase indirectly through increases in free intracellular Ca^{2+} following TRH receptor occupancy. In brain slices forskolin enhances the potency of hormones which act through receptors coupled to adenylate cyclase but enhances the efficacy of hormones which act on receptors not thought to be directly coupled to the enzyme (31). Forskolin increased the efficacy, but not the potency, of TRH for cyclic AMP accumulation (Fig. 4B) in accord with an indirect action of TRH.

The adenylate cyclase activity of cell-free preparations of GH_3 cells is unaffected by TRH, PGE_1, or EGF in the absence (22) or presence[2] of forskolin. It appears, therefore, that receptors for these hormones are not coupled to adenylate cyclase in GH_3 cells. The behavior of VIP, however, is markedly different in that enzyme activity is stimulated 4-fold (22). In intact GH_3 cells, VIP promoted a large, Ca^{2+}-dependent increase in cyclic AMP (Table 4; Fig. 5) and produced alterations in cell Ca^{2+} content (Tables 5–7). Thus the receptor for VIP may trigger adenylate cyclase activity through dual mechanisms, a direct activation and an indirect stimulation through alterations in free Ca^{2+}. It should be noted, however, that although micromolar free Ca^{2+} concentrations enhance the adenylate cyclase activity of GH_3 cell homogenates, enzyme activity due to the addition of VIP is reduced progressively as free Ca^{2+} concentrations in the assay are increased (22). Such discrepancies may reflect the disruption by the homogenization process of certain components of the adenylate cyclase system or of Ca^{2+} sequestering or transporting processes operative in the intact cell. Alternately, VIP may increase the affinity of a regulatory component of adenylate cyclase for Ca^{2+} such that maximal enzyme activity is observed at lower free Ca^{2+} concentrations in the enzyme assay. Clearly, more detailed studies with the components of the GH_3 adenylate cyclase system are needed to support the latter hypothesis.

PMA, a tumor-promoting drug, mimics the action of hormones and mitogenic substances which exert their effects through Ca^{2+} transients in many cell types (41). C6 glial tumor cells, which accumulate cyclic AMP in a Ca^{2+}-dependent manner in response to norepinephrine (20) and to cholera toxin (25), respond to PMA with a reduced Ca^{2+} content, a decreased basal cyclic AMP concentration, and a decreased ability to accumulate cyclic AMP in response to catecholamine or toxin (44). In this cell line intracellular Ca^{2+} appears to be constitutive, rather than regulatory, for cyclic AMP accumulation since neither norepinephrine nor cholera toxin promotes Ca^{2+} fluxes.[2] GH_3 cells, on the other hand, responded directly to PMA with an accumulation of cyclic AMP and with changes in cell Ca^{2+} content. Cell-associated ^{45}Ca was reduced to similar degrees by short-term (15 min) treatments with PMA, TRH, or VIP (Table 5), but after longer exposures (4½ hr) to these agents calcium uptake was reduced to a greater extent by PMA than by TRH or VIP (Table 6). Accumulation of cyclic AMP in response to PMA also differed from that to TRH or VIP in that Ca^{2+} depletion of GH_3 cells did not decrease the response to the drug (Table 4; Fig. 5). While PMA may act by promoting Ca^{2+} fluxes and cyclic AMP accumulation by mechanisms different from those of hormonal regulators, it is equally reasonable that the differential effects of PMA arise from more stable drug-receptor associations than those of the hormones examined in this study, allowing PMA effects on cyclic AMP content to occur in cells with a lower Ca^{2+} content. It is of interest that PMA stimulates the adenylate cyclase activity of GH_3 cell homogenates (22). The stimulation provided by PMA is equivalent to that provided by micromolar free Ca^{2+} concentrations and is eliminated by the addition of such Ca^{2+} concentrations to the enzyme assay. Experiments are in progress to determine whether PMA can promote the mobilization of Ca^{2+} from particulate to supernatant fractions from such cell types as GH_3 or C6.

The relationship of hormone-stimulated, Ca^{2+}-dependent accumulation of cyclic AMP to the specific functions performed by cells of the anterior pituitary remains to be defined. However, in light of the evidence favoring the involvement of both Ca^{2+} and cyclic AMP in prolactin secretion and/or synthesis, a Ca^{2+}-dependent form of adenylate cyclase may represent one pathway by which prolactin secretion and/or synthesis in response to various hormones is regulated or facilitated. In our studies, increases in cyclic AMP in Ca^{2+}-restored GH_3 cells following TRH, EGF, or PGE_1 were never greater than 3-fold and were clearly of short duration, whereas responses to VIP, PMA, and cholera toxin were of a greater magnitude. Similarly, when other workers have compared these agents with respect to capacity to stimulate prolactin synthesis or secretion, the agents were not found to be equivalent. Whereas TRH promotes both prolactin synthesis and secretion, PGE_1 (3) and EGF (33, 34) appear to exert effects primarily on prolactin synthesis. In contrast, VIP (3) and cholera toxin (6) produce greater effects on prolactin secretion but smaller effects on prolactin synthesis as compared with those of TRH. PMA is at least as effective as TRH in promoting both processes (35). It should be noted that Dannies *et al.* (4) found a correlation between the concentrations of TRH which stimulate cyclic AMP accumulation and those which enhance prolactin release; TRH concentrations which stimulated prolactin synthesis, however, did not correlate well with those which increase cyclic AMP. It therefore seems reasonable to propose that those agents which produce large elevations in cyclic AMP content will also produce greater effects on prolactin secretion. It is anticipated that investigations of prolactin synthesis and secretion in cells with defined intracellular Ca^{2+} and cyclic AMP concentrations will provide evidence for or against a requirement for the Ca^{2+}-dependent adenylate cyclase in these pituitary functions.

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REFERENCES

1. Labrie, F., P. Borgeat, A. Lemay, S. Lemaire, N. Barden, J. Drouin, I. Lemaire, P. Jolicoeur, and A. Belanger. Role of cyclic AMP in the action of hypothalamic regulatory hormones. *Adv. Cyclic Nucleotide Res.* **5**:787–801 (1975).
2. Tashjian, A. H., Jr. Clonal strains of hormone-producing pituitary cells. *Methods Enzymol.* **58**:527–535 (1979).
3. Gourdji, D., D. Bataille, N. Vauclin, D. Grouselle, G. Rosselin, and A. Tixier-Vidal. Vasoactive intestinal polypeptide (VIP) stimulates prolactin (PRL) release and cAMP production in a rat pituitary cell line (GH_3/B_6): additive effects of VIP and TRH on prolactin release. *F. E. B. S. Lett.* **104**:165–168 (1979).
4. Dannies, P. S., K. M. Gautvik, and A. H. Tashjian, Jr. A possible role of cyclic AMP in mediating the effects of thyrotropin-releasing hormone on prolactin release and on prolactin and growth hormone synthesis in pituitary cells in culture. *Endocrinology* **98**:1147–1159 (1976).
5. Gautvik, K. M., E. Walaas, and O. Walaas. Effect of thyroliberin on the concentration of adenosine 3′:5′-phosphate and on the activity of adenosine 3′:5′-phosphate-dependent protein kinase in prolactin-producing cells in culture. *Biochem. J.* **162**:379–386 (1977).
6. Dannies, P. S., and A. H. Tashjian, Jr. Action of cholera toxin on hormone synthesis and release in GH cells: evidence that adenosine 3′:5′-monophosphate does not mediate the decrease in growth hormone synthesis caused by thyrotropin-releasing hormone. *Endocrinology* **106**:1532–1536 (1980).
7. Gershengorn, M. C. Thyrotropin releasing hormone: a review of the mechanisms of acute stimulation of pituitary hormone release. *Mol. Cell. Biochem.* **45**:163–179 (1982).
8. Gautvik, K. M., and A. H. Tashjian, Jr. Effects of cations and colchicine on the release of prolactin and growth hormone by functional pituitary tumor cells in culture. *Endocrinology* **93**:793–799 (1973).
9. Gautvik, K. M., and A. H. Tashjian, Jr. Effects of Ca^{2+} and Mg^{2+} on secretion and synthesis of growth hormone and prolactin by clonal strains of pituitary cells in culture. *Endocrinology* **92**:573–583 (1973).
10. White, B. A., L. R. Bauerle, and F. C. Bancroft. Calcium specifically stimulates prolactin synthesis and messenger RNA sequences in GH_3 cells. *J. Biol. Chem.* **256**:5942–5945 (1981).
11. Dufy, B., J.-D. Vincent, H. Fleury, P. DuPasquier, D. Gourdji, and A. Tixier-Vidal. Membrane effects of thyrotropin-releasing hormone and estrogen shown by intracellular recording from pituitary cells. *Science (Wash. D. C.)* **204**:509–511 (1979).
12. Ozawa, A., and N. Kimura. Membrane potential changes caused by thyrotropin-releasing hormone in the clonal GH_3 cell and their relationship to secretion of pituitary hormone. *Proc. Natl. Acad. Sci. U. S. A.* **76**:6017–6020 (1979).
13. Taraskevich, P. S., and W. W. Douglas. Electrical behavior in a line of anterior pituitary cells (GH cells) and the influence of the hypothalamic peptide, thyrotropin releasing factor. *Neuroscience* **5**:421–431 (1980).
14. Gershengorn, M. C. Thyrotropin releasing hormone stimulation of prolactin release: evidence for a membrane potential-independent Ca^{2+}-dependent mechanism of action. *J. Biol. Chem.* **255**:1801–1803 (1980).
15. Tan, K.-N., and A. H. Tashjian, Jr. Receptor-mediated release of plasma membrane-associated calcium and stimulation of calcium uptake by thyrotropin-releasing hormone in pituitary tumor cells in culture. *J. Biol. Chem.* **256**:8994–9002 (1981).
16. Rebecchi, M. J., R. H. Gerry, and M. C. Gershengorn. Thyrotropin-releasing hormone causes loss of cellular calcium without calcium uptake by rat pituitary cells in culture: studies using arsenazo III for direct measurement of calcium. *J. Biol. Chem.* **257**:2751–2752 (1982).
17. Drust, D. S., C. A. Sutton, and T. F. J. Martin. Thyrotropin-releasing hormone and cyclic AMP activate distinctive pathways of protein phosphorylation in GH pituitary cells. *J. Biol. Chem.* **257**:3306–3312 (1982).
18. Drust, D. S., and T. F. J. Martin. Thyrotropin-releasing hormone rapidly and transiently stimulates cytosolic calcium-dependent protein phosphorylation in GH_3 pituitary tumor cells. *J. Biol. Chem.* **257**:7566–7573 (1982).
19. Schwabe, U., Y. Ohga, and J. W. Daly. The role of calcium in the regulation of cyclic nucleotide levels in brain slices of rat and guinea pig. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **302**:141–151 (1978).
20. Brostrom, M. A., C. O. Brostrom, and D. J. Wolff. Calcium dependence of hormone-stimulated cAMP accumulation in intact glial tumor cells. *J. Biol. Chem.* **254**:7548–7557 (1979).
21. Brostrom, C. O., M. A. Brostrom, and D. J. Wolff. Calcium-dependent adenylate cyclase from rat cerebral cortex: reversible activation by sodium fluoride. *J. Biol. Chem.* **252**:5677–5685 (1977).
22. Brostrom, M. A., L. A. Brotman, and C. O. Brostrom. Calcium-dependent adenylate cyclase of pituitary tumor cells. *Biochim. Biophys. Acta* **721**:227–235 (1982).
23. Wolff, D. J., P. G. Poirier, C. O. Brostrom, and M. A. Brostrom. Divalent cation binding properties of bovine brain Ca^{2+}-dependent regulator protein. *J. Biol. Chem.* **252**:4108–4117 (1977).
24. Hayashi, I., and G. H. Sato. Replacement of serum by hormones permits growth of cells in a defined medium. *Nature (Lond.)* **259**:132–134 (1976).
25. Brostrom, M. A., C. O. Brostrom, S.-C. Huang, and D. J. Wolff. Cholera toxin-stimulated cyclic AMP accumulation in glial tumor cells: modulation by Ca^{2+}. *Mol. Pharmacol.* **20**:59–67 (1981).
26. Harper, J. F., and G. Brooker. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′-*O*-acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.* **1**:207–218 (1975).
27. Steiner, A. L., C. W. Parker, and D. M. Kipnis. Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides. *J. Biol. Chem.* **247**:1106–1113 (1972).
28. Brostrom, C. O., and D. J. Wolff. Calcium-dependent cyclic nucleotide phosphodiesterase from glial tumor cells. *Arch. Biochem. Biophys.* **165**:715–727 (1974).
29. Sedmak, J. J., and S. F. Grossberg. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Anal. Biochem.* **79**:544–552 (1977).
30. Seamon, K. B., W. Padgett, and J. W. Daly. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. U. S. A.* **78**:3363–3367 (1981).
31. Daly, J. W., W. Padgett, and K. B. Seamon. Activation of cyclic AMP-generating systems in brain membranes and slices by the diterpene forskolin: augmentation of receptor-mediated responses. *J. Neurochem.* **38**:532–544 (1982).
32. Gautvik, K. M., and M. Kriz. Effects of prostaglandins on prolactin and growth hormone synthesis and secretion in cultured rat pituitary cells. *Endocrinology* **98**:352–358 (1976).
33. Johnson, L. K., J. D. Baxter, I. Vlodavsky, and D. Gospodarowicz. Epidermal growth factor and expression of specific genes: effects on cultured rat pituitary cells are dissociable from the mitogenic response. *Proc. Natl. Acad. Sci. U. S. A.* **77**:394–398 (1980).
34. Schonbrunn, A., M. Krasnoff, J. M. Westendorf, and A. H. Tashjian, Jr. Epidermal growth factor and thyrotropin-releasing hormone act similarly on a clonal pituitary cell strain: modulation of hormone production and inhibition of cell proliferation. *J. Cell Biol.* **85**:786–797 (1980).
35. Osborne, R., and A. H. Tashjian, Jr. Tumor-promoting phorbol esters affect production of prolactin and growth hormone by rat pituitary tumor cells. *Endocrinology* **108**:1164–1170 (1981).
36. Gill, D. M. Mechanism of action of cholera toxin. *Adv. Cyclic Nucleotide Res.* **8**:85–118 (1977).
37. Vaughan, M., and J. Moss. Mechanism of action of choleragen. *J. Supramol. Struct.* **8**:473–488 (1978).
38. Cassel, D., and T. Pfeuffer. Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Natt. Acad. Sci. U. S. A.* **75**:2669–2673 (1978).
39. Gill, D. M., and R. Meren. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* **75**:3050–3054 (1978).
40. Schatzmann, H. J. Active calcium transport and Ca^{2+}-activated ATPase in human red cells, in *Current Topics in Membrane Transport* (F. Bronner and A. Kleinzeller, eds.), Vol. 6. Academic Press, New York, 125–168 (1975).
41. Blumberg, P. M. *In vitro* studies on the mode of action of the phorbol esters, potent tumor promoters. *CRC Crit. Rev. Toxicol.* **8**:153–234 (1980).
42. Gershengorn, M. C., M. J. Rebecchi, E. Geras, and C. O. Arevalo. Thyrotropin-releasing hormone (TRH) action in mouse thyrotropic tumor cells in culture: evidence against a role for adenosine 3′-5′-monophosphate as a mediator of TRH-stimulated thyrotropin release. *Endocrinology* **107**:665–670 (1980).
43. Hinkle, P. M., and A. H. Tashjian, Jr. Adenylyl cyclase and cyclic nucleotide phosphodiesterases in GH-strains of rat pituitary cells. *Endocrinology* **100**:934–944 (1977).
44. Brostrom, M. A., C. O. Brostrom, L. A. Brotman, C.-S. Lee, D. J. Wolff, and H. M. Geller. Alterations of glial tumor cell Ca^{2+} metabolism and Ca^{2+}-dependent cAMP accumulation by phorbol myristate acetate. *J. Biol. Chem.* **257**:6758–6765 (1982).

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