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Mechanisms of Action of Inhibitors of Prolactin Secretion in GH_3 Pituitary Cells. I. Ca^{2+}-Dependent Inhibition of Amino Acid Incorporation

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SUMMARY

Post-transcriptional protein synthesis by GH_3 cloned pituitary cells, which secrete prolactin and growth hormone, is dependent on Ca^{2+}. The effects of antagonists of prolactin secretion were examined on overall protein synthesis in GH_3 cells as a function of cellular Ca^{2+} depletion and restoration at varying concentrations of extracellular Ca^{2+}. Leucine incorporation by Ca^{2+}-depleted cells during short incubations was reduced by 80–90%. Trifluoperazine at micromolar concentrations inhibited leucine incorporation in a Ca^{2+}-dependent manner. The extent of inhibition varied with extracellular Ca^{2+} concentration and was fully reversed at higher Ca^{2+} concentrations. Similar patterns of inhibition of leucine incorporation were observed with nifedipine, verapamil, calmidazolium, chlorpromazine, bromocriptine, ergotamine, and the (+)- and (−)-isomers of butaclamol, but dopamine, apomorphine, and chlorpromazine sulfoxide were not inhibitory. Muscarinic agonists decreased incorporation in a Ca^{2+}-dependent manner, but lesser degrees of inhibition were obtained. Inhibitions were observed for a broad spectrum of polypeptide species, could not be explained by effects on Mg^{2+} availability or amino acid uptake, and were rapidly and fully reversed by Ca^{2+}. Production of prolactin and growth hormone was decreased by secretory inhibitors to the same extent and with the same Ca^{2+} concentration dependence as was observed for inhibition of amino acid incorporation. It is proposed that these substances inhibit protein synthesis in GH_3 cells through alterations in intracellular Ca^{2+} metabolism rather than through mechanisms mediated by calmodulin or dopamine receptors.

Many neurotransmitters, hormones, and other biologically active substances exert their actions by altering the free intracellular concentration of Ca^{2+}, by mobilizing internally sequestered Ca^{2+}, and/or by stimulating plasmalemmal transport of the cation. Changes in free intracellular Ca^{2+} concentration, through interaction with cellular receptor proteins such as calmodulin, affect a wide range of cellular processes including glycogenolysis, muscle contraction, cell motility, and secretion.

GH_3 cloned pituitary tumor cells, like cells of the normal anterior pituitary, produce action potentials dependent in large part upon Ca^{2+} influx (1, 2) and produce prolactin and growth hormone (3). Transcription of messenger RNA for prolactin in these cells is regulated by Ca^{2+} (4). TRH increases both the rate of prolactin secretion and the frequency of action potential generation resulting in increased entry of Ca^{2+} from the extracellular fluid. This secretagogue also mobilizes Ca^{2+} from intracellular storage sites (5). Depolarization by high extracellular K^+ results in increased Ca^{2+} uptake through voltage-dependent Ca^{2+} channels (6) and in stimulation of prolactin release (7). Both TRH and high K^+-stimulated prolactin secretion are inhibited by reduction in extracellular Ca^{2+} and by the Ca^{2+} channel blockers, verapamil, nifedipine, and Co^{2+}. Verapamil also decreases the rate of prolactin release and the rate of rise of the action potential in nonstimulated cells (8).

Certain neurotransmitters are recognized to function as inhibitors of prolactin secretion. Anterior pituitary cells possess a high affinity, saturable, and stereoselective dopaminergic binding site, and the potencies of dopaminergic agonists to suppress hormone secretion correlate well with their binding affinities for this receptor (9). GH_3 cells, however, neither respond to low (nM) concentrations of dopamine agonists (10) nor have high affinity dopamine receptors (11). Conversely, GH_3 cells resemble normal pituitary cells in that they possess a lower affinity binding site for dopaminergic ligands that lacks the stereospecificity and characteristic selectivity of dopaminergic receptors (9, 12, 13). Higher concentrations (μM) of dopamine agonists and of dopamine antagonists suppress prolactin secretion by both cell types (10), possibly through an action at the low affinity receptor site and/or through antagonism of calmodulin-dependent processes. Binding studies have

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ABBREVIATIONS: TRH, thyrotropin-releasing hormone; MEM, modified Eagle's medium; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

also revealed sites specific for muscarinic agonists in anterior pituitary cells (14). Cholinergic agonists have been reported to decrease prolactin secretion in intact pituitary (15), dispersed anterior pituitary cells (16), and GH_3 cells (16, 17). That dopamine agonists and antagonists and cholinergic agonists also act to regulate the synthesis of prolactin remains to be determined.

The mechanism(s) through which these agents influence the rate of hormonal secretion or other pituitary functions is not established, although decreases in cAMP have been suggested to mediate the actions of both cholinergic (17) and dopaminergic (18) agonists. It is equally likely, however, that alterations in calcium pools are involved in the mechanism through which these neurotransmitter substances act. It is known, for example, that the dopamine antagonist, trifluoperazine, at high concentrations decreases depolarization-induced Ca^{2+} uptake in rat pituitary tissue (19) and blocks Ca^{2+}-dependent action potentials in GH_3 cells (20).

A fruitful approach for examining the effects of Ca^{2+} on cell function and hormonal responsiveness involves the comparison of Ca^{2+}-depleted and Ca^{2+}-restored viable cell preparations. It was recently reported that protein synthesis in a wide variety of established tumor cell lines as well as in normal dispersed chicken embryo cells and rat liver cells is reduced markedly (80–90%) by Ca^{2+} depletion (21). Repletion with Ca^{2+} but not other physiologically occurring cations restored full activity. The effects of Ca^{2+} on stimulation of amino acid incorporation were not traceable to alterations in cAMP metabolism, amino acid uptake, protein catabolism, cell ATP or GTP content, or aminoacylation of tRNA. A Ca^{2+} requirement at the translational step was proposed. In GH_3 cells, agents that increase cytosolic free Ca^{2+} concentrations were observed to enhance incorporation of amino acids into protein (22). Protein synthesis appears, therefore, to be highly sensitive to changes in intracellular Ca^{2+} concentrations.

The present study was undertaken to determine whether various inhibitors of prolactin secretion alter the Ca^{2+} requirement for protein synthesis in GH_3 cells. These substances were found to decrease amino acid incorporation in a manner dependent on the concentration of extracellular Ca^{2+}. It is proposed that these agents exert effects to reduce the availability of Ca^{2+} at key regulatory sites within the cell. Evidence is provided in this report and in the accompanying paper (23) that several of these inhibitors act to prevent Ca^{2+} uptake through the voltage-dependent Ca^{2+} channel.

Experimental Procedures

Materials. Ham's F-10 medium, Joklik's MEM, fetal bovine serum, and horse serum were purchased from Grand Island Biological Co. (Grand Island, NY). (+)- and (−)-butaclamol hydrochloride were purchased from Research Biochemicals Inc. (Wayland, MA). Trifluoperazine dihydrochloride was provided by Smith, Kline and French Laboratories (Philadelphia, PA), and chlorpromazine sulfoxide was provided by Dr. A. Manian (National Institute of Mental Health, Bethesda, MD). Reagents for growth hormone and prolactin radioimmunoassay were provided by the National Institute of Arthritis, Metabolism, and Digestive Diseases Hormone Distribution Program, and IgGSorb was obtained from the Enzyme Center (Boston, MA). L-[^{35}S]Methionine (1070 Ci/mmol) and L-[4,5-^{3}H]leucine (58 Ci/mmol) were purchased from International Chemical and Nuclear Corp. (Cleveland, OH). [^{125}I] Prolactin (46 μCi/μg) was obtained from New England Nuclear (Boston, MA). Molecular mass markers for polyacrylamide gel electrophoresis were purchased from Pharmacia Fine Chemicals (Piscataway, NJ) and EGTA was purchased from Fisher Scientific Co. (Pittsburgh, PA). All other reagents and drugs were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture and harvesting of GH_3 cells. The GH_3 strain of rat pituitary tumor cells was obtained from the American Type Culture Collection and was maintained in monolayer culture as described previously (3). Experimental cultures were grown at 37° in glass roller bottles in Ham's F-10 medium supplemented with 12.5% horse serum and 5% fetal calf serum as previously detailed (22). Cells were detached from growth surfaces with a plastic scraper. The cell suspension was centrifuged for 5 min at 600 × *g* at room temperature and the supernatant fluid was discarded. Ca^{2+}-depleted cells were prepared by resuspending the cell pellet in 50 ml of Joklik's low Ca^{2+} MEM containing 1 mM EGTA and 25 mM Hepes (pH 7.4) followed by recentrifugation of the cell suspension for 5 min at 600 × *g*. The resuspension and centrifugation steps were then repeated, and the final cell pellet was resuspended in buffered Joklik's MEM containing 1 mM EGTA and 1 mg/ml of fatty acid-free bovine albumin to a final cell protein content of 0.1–0.3 mg/ml. Ca^{2+}-restored cells were prepared by adding $CaCl_2$ to a portion of the depleted cells. Ca^{2+}-depleted cells exposed to 1.5 mM extracellular $CaCl_2$ were observed to contain 5- to 7-fold more Ca^{2+} than did Ca^{2+}-depleted cells exposed to EGTA (24). Viability of cell preparations was determined by dye uptake studies and replating procedures as described previously (25). By these criteria, cells were routinely 90–95% viable.

Determination of amino acid incorporation. The incorporation of radioactive amino acid into trichloroacetic acid-precipitable, alkali-stable material was considered to represent incorporation into protein. Aliquots (0.5 ml) of Ca^{2+}-depleted cell preparations were pretreated at 37° in glass vials with or without Ca^{2+} and drugs or other additives for 20 min. Most drugs were prepared in buffered saline and added in 10-μl volumes to vials. Bromocriptine, ergotamine, and nifedipine were dissolved in dimethyl sulfoxide and were either added directly to vials in 2-μl volumes or diluted in buffered saline prior to addition. The addition of equivalent concentrations of dimethyl sulfoxide without drug did not affect either amino acid incorporation or hormone production. Unless otherwise specified, incubations were conducted in triplicate with 10^6 cpm of [^{3}H]leucine for 90 min at 37°. Incorporation of label into trichloroacetic acid-insoluble material was determined as described previously (21). Values for replicate samples were routinely within 5% of each other. Standard errors are indicated for the data provided in tabular form. Values for data presented in figures were averaged without further statistical analysis.

Determination of amino acid uptake. Aliquots (3 ml) of Ca^{2+}-depleted cell preparations were preincubated at 37° for 20 min with additives. [^{3}H]Leucine (5 × 10^6 cpm/ml) was then added. At various times 300-μl aliquots were withdrawn in duplicate, added to 1.5-ml polypropylene tubes containing 500 μl of dinonyl phthalate/silicon oil (1:1, v/v) layered over 200 μl of 10% trichloroacetic acid, and centrifuged for 30 sec in a Beckman Microfuge. The top two layers were carefully removed and a 100-μl aliquot of the lower phase was withdrawn for analysis of trichloroacetic acid-soluble radioactivity. The remaining liquid was then aspirated and the tip of the tube containing the protein precipitate was cut off with a razor blade and placed in a glass vial. The pellet was solubilized by addition of formic acid and aliquots were analyzed for trichloroacetic acid-insoluble radioactivity. To assess the contribution of extracellular [^{3}H]leucine, unlabeled leucine (5 mM) was added to additional samples which were immediately centrifuged through oil into trichloroacetic acid. Values obtained constituted the blanks and were subtracted from experimental values.

Measurement of growth hormone and prolactin production. The increase in total hormone content of cell suspensions (cells with extracellular medium) over a defined incubation period was considered to be a measure of growth hormone or prolactin production since hormone is stable in culture medium (26) and is not degraded within cells to a significant extent (27). Cell suspensions (0.5 ml) in plastic

tubes were disrupted by the addition of 0.5 ml of ice-cold distilled water followed by sonic irradiation for 15 sec. Lysates were immediately frozen. Growth hormone was iodinated and prolactin and growth hormone concentrations were measured by radioimmunoassay using procedures recommended by the National Institute of Arthritis, Metabolism, and Digestive Diseases. Antigen-antibody complex was precipitated with a 1:5 suspension of formalin-fixed *Staphylococcus aureus* (IgGSorb). For each experimental value, triplicate biological samples were prepared, and each was assayed for hormone content in duplicate.

Miscellaneous procedures. Calcium solutions were standardized by atomic absorption spectrophotometry. Protein determinations were conducted by the method of Sedmak and Grossberg (28) using bovine serum albumin as standard. Polyacrylamide gel electrophoresis was conducted using 12.5% acrylamide gels as described by Laemmli (29).

Results

Effect of Ca^{2+} and various modifiers of prolactin secretion on GH_3 cell amino acid incorporation. In previous work (22) the rates of both prolactin production and overall protein synthesis in GH_3 cells were found to be comparably dependent on the concentration of extracellular Ca^{2+} and to be similarly affected by various stimulators of prolactin production, including TRH and phorbol myristate acetate. In the present study, a series of inhibitors of GH_3 prolactin and growth hormone production, although of widely varying pharmacologic classification, was found to inhibit protein synthesis in an apparently similar manner. The incorporation of [^{3}H]leucine into protein for cells incubated in Joklik's MEM adjusted to 1 mM EGTA was highly sensitive to the addition of extracellular Ca^{2+} (Fig. 1). Rates of incorporation increased in a graded fashion as a function of increasing extracellular Ca^{2+} concentration to a maximal 10-fold stimulation at 1 mM added cation. Although the incubation conditions were somewhat different from those in the previous study (22), the results of control incubations are qualitatively and quantitatively similar.

Various agents known to interact with dopamine receptors and to influence prolactin secretion were tested for effects on leucine incorporation. Dopamine, at concentrations ranging from 1 nM to 1 mM, was without effect. However, the dopamine antagonist and reputed calmodulin antagonist, trifluoperazine, inhibited [^{3}H]leucine incorporation in a Ca^{2+}-dependent manner (Fig. 1). Below 1 mM added extracellular Ca^{2+}, trifluoperazine (1, 3, and 10 μM) significantly decreased incorporation of leucine as compared to the incorporation in incubations without drug. The maximal extent of inhibition by 10 μM drug was about 90%. By contrast, at the concentration of extracellular Ca^{2+} at which the maximal rate of protein synthesis prevailed, inhibition by 1 or 3 μM drug was abolished. Concentrations of extracellular Ca^{2+} in excess of 1.1 mM were required to abolish completely the inhibition occurring at 10 μM trifluoperazine. Chlorpromazine inhibited amino acid incorporation in a similar fashion (Fig. 2A). Chlorpromazine sulfoxide, an analog of chlorpromazine which lacks antipsychotic activity (30), the ability to bind to dopamine receptors (31), and calmodulin antagonist activity (31), was not an effective inhibitor of protein synthesis even at much higher concentrations (Table 1). Other dopamine antagonists, haloperidol and (+)-butaclamol, at concentrations ranging from 1 to 10 μM also inhibited [^{3}H]leucine incorporation significantly at 0.9 mM added Ca^{2+} but not at 1.5 mM (Table 1). It was also of interest that (−)-butaclamol, which is relatively inactive as a dopamine receptor antagonist, was more effective in reducing leucine incorporation than (+)-butaclamol.

Various types of ergoline dopaminergic agonists were also found to reduce leucine incorporation at low but not at high extracellular Ca^{2+} concentrations. For example, 10 μM bromocriptine altered the Ca^{2+} concentration dependence of leucine incorporation such that no stimulation was observed until added cation exceeded 0.9 mM, an 85% reduction relative to values found for untreated controls (Fig. 2B). Ergotamine added at 1 and 10 μM reduced values for leucine incorporation by approximately 40 and 90%, respectively (Table 1). In contrast, apomorphine, a classic dopamine agonist, exerted no effects on

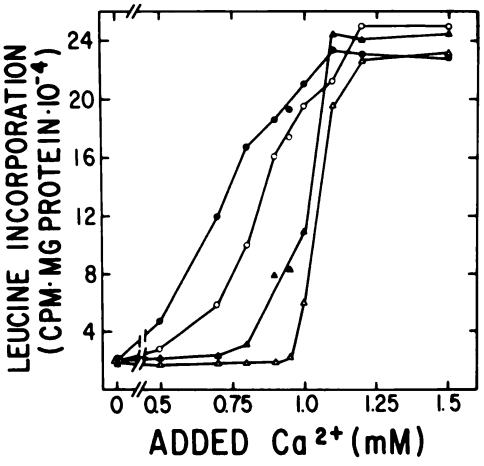

Fig. 1. Effect of trifluoperazine on the Ca^{2+} concentration dependence of leucine incorporation by GH_3 cells. Ca^{2+}-Depleted cells were suspended in Joklik's MEM containing 25 mM Hepes, pH 7.4, 1 mM EGTA, and 1 mg/ml of fatty acid free-bovine serum albumin. Cell suspensions were pretreated for 20 min at 37° with the indicated concentrations of added $CaCl_2$ with no addition (●), 1 μM trifluoperazine (○), 3 μM trifluoperazine (▲), or 10 μM trifluoperazine (△). [^{3}H]Leucine (10^6 cpm/0.5 ml of cell suspension) was added, and incorporation of radioactivity into trichloroacetic acid-precipitable protein was determined after 90 min of incubation. Results of one experiment are presented. Findings have been reproduced in two separate experiments.

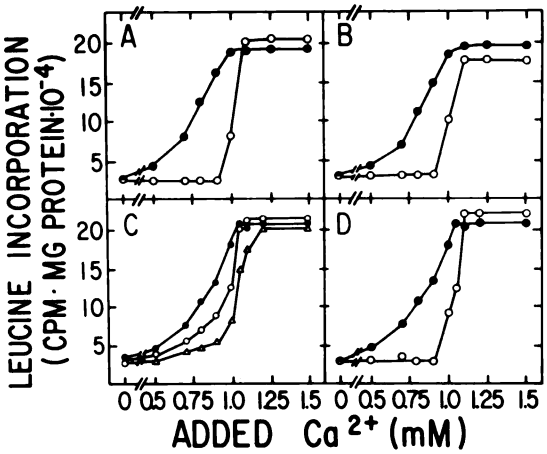

Fig. 2. Effects of chlorpromazine, bromocriptine, acetylcholine, and nifedipine on the Ca^{2+} concentration dependence of leucine incorporation by GH_3 cells. Ca^{2+}-depleted cells were pretreated for 20 min with the indicated concentrations of added $CaCl_2$ with (A–D) no addition (●), or with (A) 10 μM chlorpromazine (○), (B) 10 μM bromocriptine (○), (C) 100 nM (○) or 1 μM acetylcholine (△), or (D) 1 μM nifedipine (○). Incorporation of [^{3}H]leucine into trichloroacetic acid-insoluble protein was determined after 90 min of incubation. Results of a single experiment are provided. Findings have been reproduced in three separate experiments.

TABLE 1

Ca^{2+}-dependent inhibition by selected agents of leucine incorporation by GH_3 cells

Ca^{2+}-depleted GH_3 cells were incubated in buffered Joklik's MEM with 1 mM EGTA, 1 mg/ml of fatty acid-free bovine serum albumin, and the indicated concentrations of inhibitors and added $CaCl_2$. Following a 20-min pretreatment, [^{3}H]leucine (10^6 cpm/0.5 ml of cell suspension) was added and incorporation into trichloroacetic acid-precipitable protein was measured after 90 min of incubation. Results are expressed as the percentage of incorporation into untreated controls ± SE of triplicate samples from a single experiment and have been reproduced in three separate experiments.

Additive		Leucine incorporation (% of control values)	
		0.9 mM Ca^{2+}	1.5 mM Ca^{2+}
None		100	100
Haloperidol	(5 μM)	34.1 ± 0.6	103 ± 1
(+)-Butaclamol	(1 μM)	87.0 ± 0.6	92.5 ± 1.6
	(3 μM)	71.2 ± 1.0	97.4 ± 0.6
	(10 μM)	23.5 ± 0.2	86.1 ± 1.6
(−)-Butaclamol	(1 μM)	54.0 ± 1.4	96.1 ± 1.4
	(3 μM)	19.6 ± 1.1	99.1 ± 0.8
	(10 μM)	14.0 ± 0.2	104 ± 1
Chlorpromazine sulfoxide	(10 μM)	103 ± 1	105 ± 1
	(30 μM)	95.0 ± 1.1	98 ± 1.5
	(100 μM)	90.8 ± 0.2	108 ± 1
Ergotamine	(1 μM)	63.5 ± 1.6	nd[a]
	(3 μM)	43.1 ± 1.6	nd
	(10 μM)	19.3 ± 0.7	nd
Apomorphine	(10 μM)	106 ± 4	nd

[a] nd, not determined.

leucine incorporation. The Ca^{2+} dependence of leucine incorporation appeared to be affected in a qualitatively similar manner not only by the phenothiazines, trifluoperazine (Fig. 1), and chlorpromazine (Fig. 2A), and by bromocriptine (Fig. 2B), but also by acetylcholine (Fig. 2C) and the Ca^{2+} channel blocker, nifedipine (Fig. 2D).

The concentration dependencies of inhibition of leucine incorporation by chlorpromazine, trifluoperazine, bromocriptine, and the reputed calmodulin antagonist, calmidazolium (33), were examined for cells in medium containing either 0.9 mM or 1.5 mM Ca^{2+} (Fig. 3). At limiting Ca^{2+} concentrations, leucine incorporation was decreased significantly (30–40%) at 1 μM chlorpromazine, trifluoperazine, or bromocriptine. At 20 μM bromocriptine or trifluoperazine and at 30 μM chlorpromazine, inhibition of protein synthesis was observed at both extracellular Ca^{2+} concentrations. Calmidazolium at 3 μM inhibited amino acid incorporation in a Ca^{2+}-dependent fashion, but inhibition by higher drug concentrations was not reversed by 1.5 mM Ca^{2+}.

The effects of cholinergic agonists on the Ca^{2+} dependence of leucine incorporation noted above (Fig. 2C) were examined in more detail. Acetylcholine at 1 μM decreased amino acid incorporation at added Ca^{2+} concentrations below 1.2 mM, whereas 100 nM acetylcholine was inhibitory below 1.05 nM added Ca^{2+} (Fig. 2C). Acetylcholine, however, produced lesser degrees of inhibition than the other inhibitors. Similar effects were obtained with carbachol (data not shown). Dose-response relationships for inhibitions by acetylcholine and carbachol in cells restored with 0.9 mM Ca^{2+} are shown in Fig. 4. Half-maximal inhibition was obtained at 40 nM acetylcholine and 700 nM carbachol, and maximal inhibition (50%) occurred at 1 μM acetylcholine and 10 μM carbachol. These agents act at muscarinic cholinergic receptors, since inhibition of leucine incorporation by acetylcholine and carbachol was reversed by atropine but not by hexamethonium (Table 2). Inhibition of

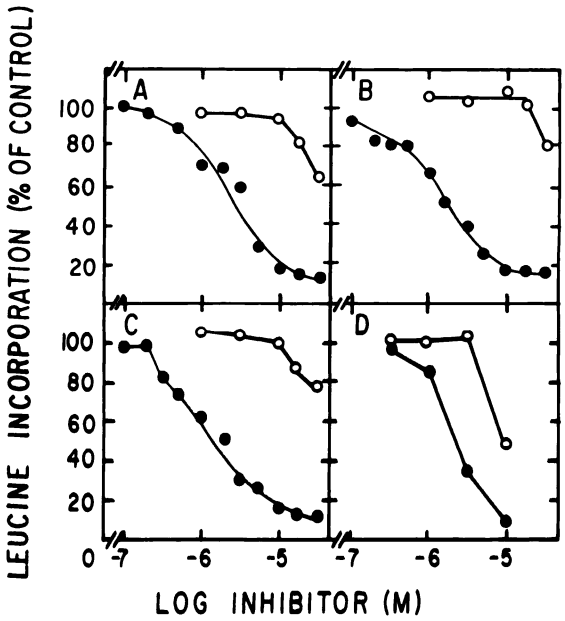

Fig. 3. Trifluoperazine, chlorpromazine, bromocriptine, and calmidazolium concentration dependencies of inhibition of leucine incorporation by GH_3 cells. Ca^{2+}-depleted cells were suspended in buffered Joklik's MEM containing 1 mM EGTA and 1 mg/ml of bovine serum albumin. Cell suspensions were pretreated at 0.9 mM (●) or 1.5 mM (○) added $CaCl_2$ and the indicated concentrations of (A) trifluoperazine, (B) chlorpromazine, (C) bromocriptine, or (D) calmidazolium. [^{3}H]Leucine was then added and incorporation of radioactivity into trichloroacetic acid-precipitable protein was measured after 90 min of incubation. Results are expressed as percentage of radioactivity incorporated by untreated control cell preparations for a single experiment. Findings have been reproduced in three separate experiments.

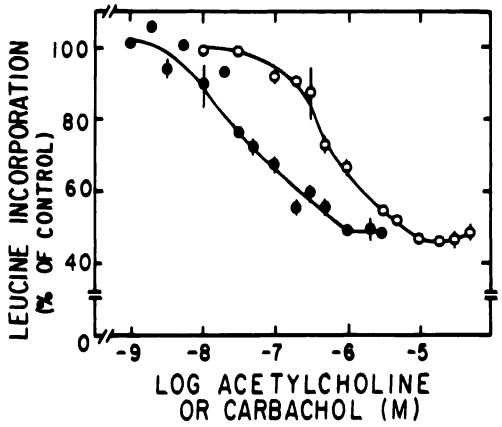

Fig. 4. Acetylcholine and carbachol concentration dependencies of inhibition of leucine incorporation by GH_3 cells. Ca^{2+}-Depleted cells were suspended in buffered Joklik's MEM containing 1 mM EGTA, 1 mg/ml of bovine serum albumin, and 0.9 mM added $CaCl_2$. Cell suspensions were pretreated with the indicated concentrations of acetylcholine (●) or carbachol (○) for 20 min. [^{3}H]Leucine was added and incorporation of radioactivity into trichloroacetic acid precipitates was determined after 90 min of incubation. Results are expressed as percentage of incorporation observed in the absence of drug ± range of values obtained for a single experiment. Findings have been reproduced in three separate experiments.

leucine incorporation by trifluoperazine or bromocriptine, in contrast, was not reversed by atropine, indicating that inhibition of protein synthesis by these agents is not mediated through interactions at muscarinic receptor sites (data not shown).

TABLE 2

Effects of cholinergic antagonists on inhibition of leucine incorporation by carbachol and acetylcholine

GH_3 cells were harvested as described in the legend for Table 1. Cells were exposed to 0.9 mM $CaCl_2$ and pretreated for 20 min with the indicated concentrations of agonists and antagonists. [^{3}H]Leucine (10^6 cpm/0.5 ml of cell suspension) was added, and incorporation of amino acid was determined after 90 min of incubation. Results are expressed as percentage of incorporation occurring in control incubations ± SE of triplicate determinations from a single experiment. Findings have been reproduced in three separate experiments.

Additive	% of Control
None	100
0.1 μM Atropine	106 ± 3
50 μM Hexamethonium	102 ± 3
10 μM Carbachol	46.8 ± 1.5
+ 0.1 μM Atropine	92.0 ± 1.3
+50 μM Hexamethonium	48.2 ± 2.0
1 μM Acetylcholine	54.4 ± 2.1
+ 0.1 μM Atropine	85.6 ± 1.9

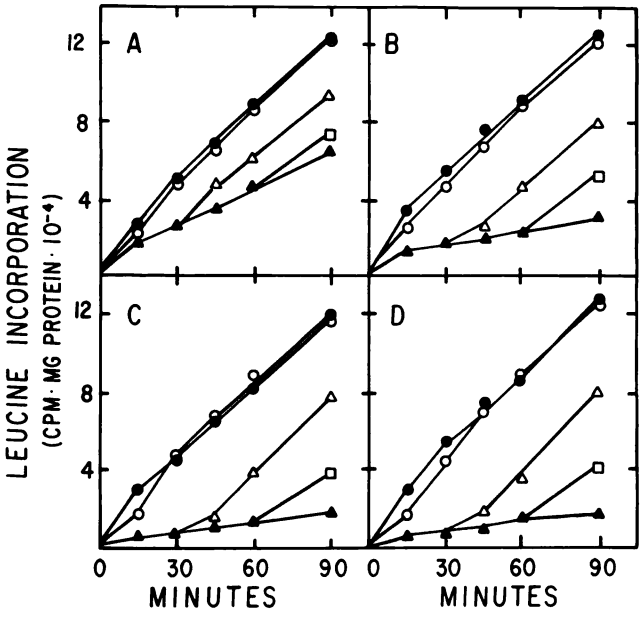

Fig. 5. Rapid reversal by Ca^{2+} of inhibition of leucine incorporation by acetylcholine, trifluoperazine, and chlorpromazine. Ca^{2+}-depleted cells were suspended in buffered Joklik's MEM containing 1 mM EGTA, 1 mg/ml of bovine serum albumin, and either 1.5 mM (●) or 0.9 mM (▲) added $CaCl_2$. Cell suspensions were pretreated for 20 min with (A) no addition, (B) 1 μM acetylcholine, (C) 10 μM trifluoperazine, or (D) 10 μM chlorpromazine. [^{3}H]Leucine was then added and incorporation of label into trichloroacetic acid precipitates was determined for the indicated incubation times. An additional 0.6 mM $CaCl_2$ was added to portions of the cell suspensions with 0.9 mM extracellular $CaCl_2$ at 0 min (○), 30 min (△), or 60 min (□) of incubation. Results of a single experiment are provided. Findings have been reproduced in two separate experiments.

The time required for Ca^{2+} to reverse inhibitions of leucine incorporation by trifluoperazine, chlorpromazine, and acetylcholine was examined (Fig. 5). Ca^{2+}-depleted cell preparations were pretreated for 20 min with 0.9 mM Ca^{2+} in the absence or presence of drugs. Portions of each cell preparation were exposed to an additional 0.6 mM Ca^{2+} at the start of the pretreatment period, at the time of [^{3}H]leucine addition, or after 30 or 60 min of incubation. Amino acid incorporation was measured at the indicated times throughout the 90-min incubation period. Linear rates of incorporation were obtained for all preparations although cells treated with drug at the lower Ca^{2+} concentration incorporated label at a significantly lower rate. This reduced rate of incorporation, however, was increased within minutes to that of untreated controls when the additional 0.6 mM $CaCl_2$ was added to the medium. It was apparent, therefore, that inhibition of protein synthesis by the three agents is rapidly and fully reversed by Ca^{2+}.

Site of action of inhibitors. Mg^{2+} is recognized to be required for the maintenance of cellular functionality and protein synthesis; however, neither the inhibition of amino acid incorporation seen upon addition of EGTA nor the reversal of inhibition resulting from restoration of the medium with Ca^{2+} is attributable to chelation of Mg^{2+} (21). Measurements of leucine incorporation conducted at various concentrations of Mg^{2+} and Ca^{2+} provided evidence that the agents described in the present study did not act to inhibit protein synthesis by limiting the availability of Mg^{2+} (Table 3). Mg^{2+} in excess of EGTA had little influence on the activity of incubations without drug that were conducted without added Ca^{2+} or at 1.5 mM Ca^{2+}. In comparable controls conducted at limiting (0.9 mM) Ca^{2+}, additional Mg^{2+} proved inhibitory, presumably due to competition by Mg^{2+} with Ca^{2+} for binding sites such as those described for the voltage-dependent Ca^{2+} channel (6). Mg^{2+} in excess of EGTA did not appear to affect leucine incorporation in incubations conducted with trifluoperazine, bromocriptine, or carbachol at 0, 0.9, or 1.5 mM added extracellular Ca^{2+}.

Although leucine uptake by GH_3 cells is not dependent on Ca^{2+} (22), it was possible that these agents inhibited leucine incorporation by decreasing uptake of the amino acid in a Ca^{2+}-dependent manner. To investigate this possibility, uptake of [^{3}H]leucine was measured after 1, 6, and 30 min of incubation in the presence and absence of acetylcholine, chlorpromazine, or bromocriptine and under conditions comparable to those for which inhibition of leucine incorporation is observed (Table 4). Radioactivity in the trichloroacetic acid-soluble fraction, which was considered to represent nonincorporated leucine, was unaffected by drug treatment. In contrast, incorporation into the trichloroacetic acid-insoluble pool was decreased by the drugs at all incubation times examined.

The possibility that these agents produced in a rapid fashion nonspecific toxic effects to which GH_3 cells were susceptible

TABLE 3

Effect of extracellular Mg^{2+} concentration on Ca^{2+}-dependent inhibition of leucine incorporation by trifluoperazine, bromocriptine, and carbachol in GH_3 cells

Ca^{2+}-depleted cells were pretreated for 20 min with $MgCl_2$, $CaCl_2$, and inhibitors at the concentrations indicated, and incorporation of leucine into trichloroacetic acid-precipitable protein was determined after 90 min of incubation with labeled amino acid (10^6 cpm/0.5 ml of cell suspension). Results are expressed as the mean ± SE of triplicate determinations from a single experiment.

Additive	Added $CaCl_2$ (mM)	Leucine incorporation (cpm × 10^{-3}/mg of protein)	
		1.0 mM $MgCl_2$	1.5 mM $MgCl_2$
None		20.0 ± 0.6	16.6 ± 0.6
	0.9	86.8 ± 2.0	46.4 ± 1.6
	1.5	199 ± 3	201 ± 3
Trifluoperazine (10 μM)		14.7 ± 0.3	14.0 ± 0.4
	0.9	16.5 ± 0.5	15.4 ± 0.2
	1.5	186 ± 1	182 ± 1
Bromocriptine (10 μM)		14.9 ± 0.1	14.7 ± 0.3
	0.9	16.0 ± 0.3	15.9 ± 0.2
	1.5	173 ± 1	172 ± 6
Carbachol (10 μM)		18.2 ± 0.1	17.5 ± 0.3
	0.9	26.0 ± 1.1	20.1 ± 0.2
	1.5	188 ± 2	185 ± 3

TABLE 4

Leucine uptake and incorporation by GH_3 cells treated with chlorpromazine, bromocriptine, or acetylcholine

Ca^{2+}-depleted cells were suspended in buffered Joklik's MEM containing 1 mM EGTA, 1 mg/ml of bovine serum albumin, 0.9 mM $CaCl_2$, and agents at the indicated concentrations. After a 20-min pretreatment, [^{3}H]leucine (5×10^6 cpm/ml) was added, and radioactivity in the trichloroacetic acid-soluble and insoluble fractions was determined as described in Experimental Procedures. The mean of duplicate incubation samples is presented. Results have been reproduced in two separate experiments.

Additive	Cell-associated radioactivity (cpm · mg protein^{-1} · 10^{-3})					
	Trichloroacetic acid-soluble			Trichloroacetic acid-insoluble		
	1 min	6 min	30 min	1 min	6 min	30 min
None	182	140	130	19.0	83.4	392
Chlorpromazine (10 μM)	188	164	131	13.8	19.7	115
Bromocriptine (10 μM)	180	153	130	13.9	22.2	132
Acetylcholine (1 μM)	189	136	147	14.5	40.2	170

only at low Ca^{2+} concentrations was also considered. Cells pretreated at 0, 0.9, and 1.5 mM Ca^{2+} with or without chlorpromazine (10 μM), bromocriptine (10 μM), or acetylcholine (1 μM) for 20 min were therefore examined for ATP content by high performance liquid chromatography (18). Concentrations of this nucleotide, however, were not found to vary significantly as a function either of extracellular Ca^{2+} or of drug treatment.

The voltage-dependent Ca^{2+} channel antagonists nifedipine and verapamil decrease prolactin secretion by clonal lines of GH cells (7, 8). Blockage of Ca^{2+} entry, therefore, represents an alternative mechanism through which secretory inhibitors could inhibit protein synthesis. The effects of nifedipine on amino acid incorporation as a function of extracellular Ca^{2+} concentration, as noted earlier (Fig. 2D), are comparable to those produced by other secretory inhibitors on this parameter. At 1 μM nifedipine, incorporation was inhibited 80% at 0.9 mM added Ca^{2+}, with the inhibition being suppressed with increasing concentrations of extracellular Ca^{2+}. The concentration dependence of the inhibitions by nifedipine and verapamil was investigated (Fig. 6). Half-maximal effects were obtained at 30 nM nifedipine and 1 μM verapamil, and maximal effects were found at 1 μM nifedipine and 10 μM verapamil. At these concentrations, nifedipine and verapamil are effective inhibitors of Ca^{2+} uptake by GH_3 cells (6). The maximal extent of inhibition seen in this experiment with both agents was about 70%. At the higher concentration of added Ca^{2+}, leucine incorporation was not decreased significantly by either drug.

Spectrum of polypeptides affected by inhibitors. To determine whether inhibitors decreased amino acid incorporation into all polypeptides being synthesized or affected the synthesis of only particular polypeptides, cells were incubated at varying extracellular Ca^{2+} with or without drugs in medium containing [^{35}S]methionine. Labeled polypeptides were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography (Fig. 7). As reported previously, Ca^{2+} depletion decreased incorporation into all polypeptides detected (21, 22). Treatment with bromocriptine, chlorpromazine, or acetylcholine at limiting Ca^{2+} also resulted in decreased labeling of all detectable polypeptide species. At high extracellular Ca^{2+}, however, the extent of labeling of polypeptides from drug treatment samples appeared identical to that from untreated controls.

GH_3 cells synthesize and secrete growth hormone and prolactin. Production of prolactin (22) and growth hormone[1] during short incubations exhibits the same Ca^{2+} concentration dependence as does amino acid incorporation into overall protein. It was of interest, therefore, to ascertain whether the agents of interest would decrease growth hormone and prolactin production in a Ca^{2+}-dependent manner. Incorporation of leucine and production of growth hormone were measured simultaneously in Ca^{2+}-depleted preparations of GH_3 cells exposed to 0, 0.9, or 1.5 mM extracellular Ca^{2+} and treated with bromocriptine, trifluoperazine, or chlorpromazine. As shown in Fig. 8, both growth hormone production and leucine incorporation were decreased in the presence of bromocriptine, trifluoperazine, or chlorpromazine, provided that incubations were conducted at limiting Ca^{2+}. Furthermore, the degree of inhibition (70%) was similar for both parameters.

The production of growth hormone and prolactin was compared for cell preparations exposed to either 0.9 mM or 1.5 mM extracellular Ca^{2+} and treated with trifluoperazine, chlorpromazine, bromocriptine, nifedipine, or acetylcholine (Table 5). Hormone production by cell suspensions (cells plus medium) was determined after a 90-min incubation. Growth hormone and prolactin production were both decreased by drugs in a dose-dependent manner at limiting Ca^{2+} concentrations. However, at 1.5 mM Ca^{2+}, hormone production was unaffected except at the highest trifluoperazine concentrations. Protein synthesis was also inhibited by high doses of trifluoperazine under these conditions (Fig. 3). Atropine reversed specifically the inhibition by carbachol or acetylcholine of growth hormone and prolactin production (data not shown).

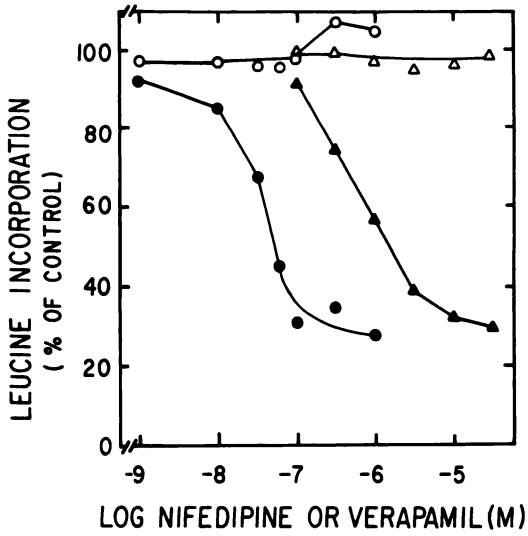

Fig. 6. Nifedipine and verapamil concentration dependencies of leucine incorporation by GH_3 cells. Ca^{2+}-depleted cells were pretreated for 20 min with either 0.9 mM (●, ▲) or 1.5 mM (○, △) $CaCl_2$ and the indicated concentrations of nifedipine (●, ○), or verapamil (▲, △). Incorporation of [^{3}H]leucine into trichloroacetic acid precipitates was determined after 90 min of incubation, and the results of a single experiment are presented. Similar results were obtained in three separate experiments.

Discussion

Protein synthesis in normal liver and chicken embryo cells and numerous established tumor cell lines, including the prolactin-secreting GH_3 cell line, is markedly dependent on Ca^{2+} (21,

[1] S. Wolfe, unpublished observations.

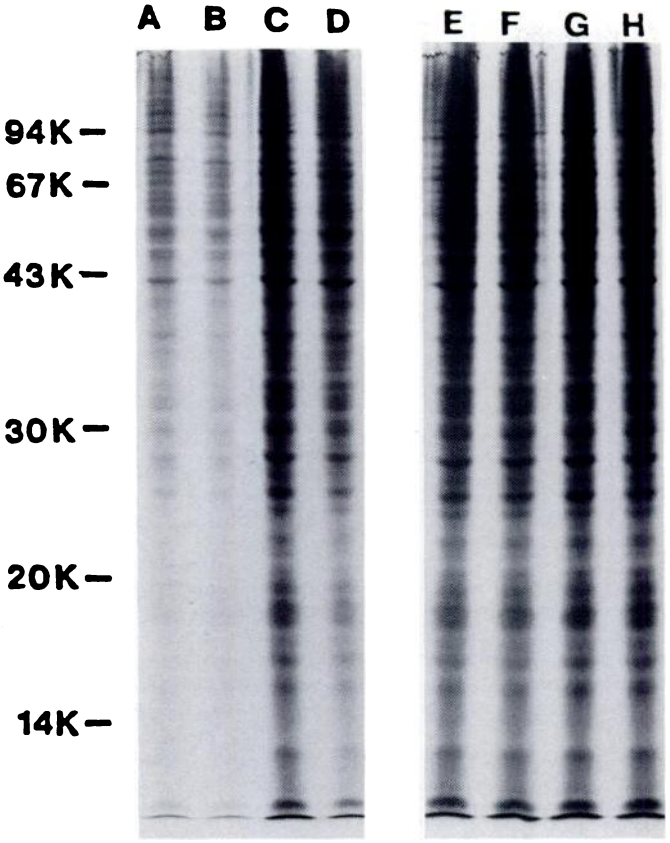

Fig. 7. Effects of chlorpromazine, bromocriptine, and acetylcholine on [^{35}S]methionine incorporation as analyzed by polyacrylamide gel electrophoresis. Ca^{2+}-depleted cells were suspended in a modified MEM lacking added Ca^{2+} or methionine and containing 25 mM Hepes, pH 7.4, and 1 mM EGTA. Samples were pretreated at 37° for 20 min with 0.9 mM (*lanes A–D*) or 1.5 mM $CaCl_2$ (*lanes E–H*), and either no addition (*lanes C* and *F*), 1 μM acetylcholine (*lanes D* and *E*), 10 μM bromocriptine (*lanes A* and *H*), or 10 μM chlorpromazine (*lanes B* and *G*). Samples were then incubated for 60 min with [^{35}S]methionine (4×10^6 cpm/ml), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography were performed as described in Experimental Procedures. The following molecular weight marker proteins were used: skeletal muscle phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

22). In the present study, drugs and neurotransmitters reported to inhibit prolactin secretion were examined for their effects on amino acid incorporation by GH_3 cells as a function of Ca^{2+} depletion and restoration. The dopamine agonists, bromocriptine and ergotamine, the phenothiazine dopamine antagonists, trifluoperazine and chlorpromazine, and cholinergic agonists were found to inhibit incorporation of leucine into overall protein in a Ca^{2+}-dependent manner without affecting cell viability. Furthermore, the Ca^{2+} concentration dependencies of inhibition were similar for these agents.

Several possible explanations could account for these findings. Inhibition of amino acid incorporation by trifluoperazine, chlorpromazine, bromocriptine, and ergotamine could result from nonspecific hydrophobic interactions and/or membrane perturbation effects since these drugs are rather lipophilic and are reported to stabilize membranes (34). This interpretation does not appear to be valid, however, since the inhibitions were invariably reversible by Ca^{2+} except at very high (20 μM or greater) drug concentrations (Fig. 3). It is unlikely that Ca^{2+} prevents or reverses association of the various agents with the membrane since the agents are effective inhibitors of prolactin secretion and of Ca^{2+}-dependent action potentials at physiologic extracellular Ca^{2+} concentrations (8, 16, 17, 20). In addition, chlorpromazine sulfoxide at 10 or 30 μM did not significantly decrease amino acid incorporation (Table 1).

Trifluoperazine, chlorpromazine, and calmidazolium bind to the Ca^{2+}·calmodulin complex and prevent activation of calmodulin-dependent enzymes *in vitro*. These agents, therefore, have been frequently used as probes for calmodulin-dependent effects in intact cells. It is unlikely, however, that inhibition of amino acid incorporation at limiting extracellular Ca^{2+} concentrations by trifluoperazine, chlorpromazine, or calmidazolium was mediated through binding to calmodulin for at least three reasons. First, although trifluoperazine, chlorpromazine, and calmidazolium inhibited protein synthesis half-maximally at 0.9 mM Ca^{2+} at approximately the same concentration (1 μM), their established affinities for binding to calmodulin vary greatly from each other (32, 33). Second, effects on leucine incorporation were observed at lower concentrations of trifluoperazine and chlorpromazine than have been reported to influence calmodulin-dependent processes (32). Finally, ergotamine and bromocriptine inhibited amino acid incorporation to the same extent and with the same Ca^{2+} dependence as trifluoperazine and chlorpromazine, yet neither of these dopamine agonists has been reported to possess calmodulin-antagonistic activity *in vitro*.

GH_3 cells, unlike normal pituitary tissue, neither possess high affinity dopamine receptors nor respond to nanomolar concentrations of dopamine agonists. A separate site to which dopaminergic agonists and antagonists bind with affinities in the micromolar range, however, has been identified in GH_3 cells and normal anterior pituitary cells (9, 11). The binding affinities of a number of dopamine agonists and antagonists at this low affinity site correlate well with the concentrations at which these agents inhibit both amino acid incorporation and prolactin secretion (10). Furthermore, the active and inactive isomers of butaclamol bind to this site with comparable affinities (11) and inhibit protein synthesis (Table 1) and prolactin release (10) at equivalent concentrations. Finally, the dopamine agonist, apomorphine, which possesses a 100-fold lower binding affinity for this receptor, did not significantly inhibit amino acid incorporation (Table 1). Therefore, inhibition of amino acid incorporation and of prolactin secretion by these agents could be mediated through binding to this lower affinity receptor site.

TRH, a Ca^{2+}-mobilizing hormone, has been shown to increase both prolactin secretion (5) and amino acid (22). Decreased prolactin secretion and amino acid incorporation in the presence of drugs used in this study could, therefore, result from reduced availability of Ca^{2+} at key regulatory sites within the cell. Inhibitions of amino acid incorporation were clearly dependent on the extracellular concentration of Ca^{2+}. As further support for this hypothesis, trifluoperazine is reported to block Ca^{2+}-dependent action potentials in GH_3 cells (20) and to decrease high K^+-induced Ca^{2+} uptake in both rat synaptosomes (35) and rat anterior pituitary cells (19). In addition, verapamil and nifedipine were observed in this study to decrease amino acid incorporation at concentrations at which these agents are effective Ca^{2+} channel antagonists (6). Leucine incorporation was inhibited to the same extent and within the same range of extracellular Ca^{2+} concentrations as was observed

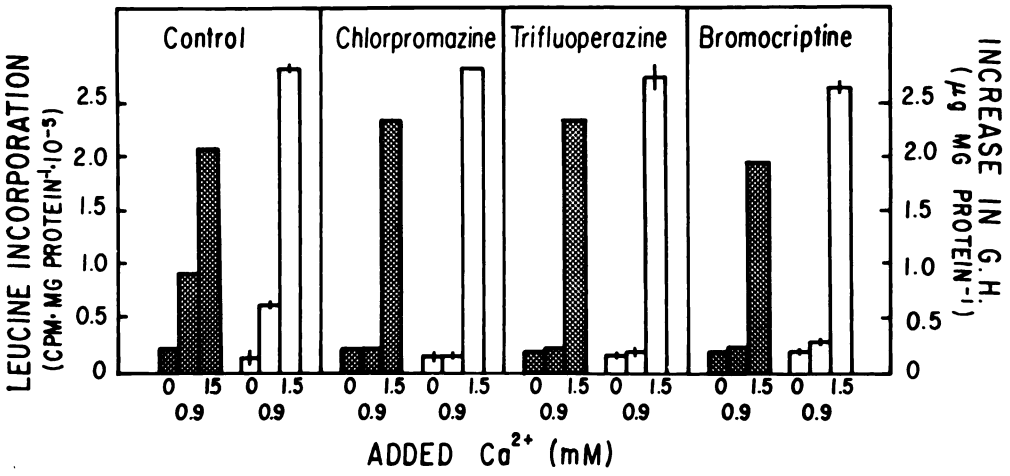

Fig. 8. Inhibition of growth hormone production and leucine incorporation by chlorpromazine, trifluoperazine, and bromocriptine, Ca^{2+}-depleted cells were suspended in buffered Joklik's MEM containing 1 mM EGTA and 1 mg/ml of bovine serum albumin. Cell suspensions were pretreated for 20 min with 0, 0.9 or 1.5 mM $CaCl_2$ and with 10 μM bromocriptine, 10 μM chlorpromazine, or 10 μM trifluoperazine. [^{3}H]Leucine was then added to portions of the cell suspensions, and incorporation of radioactivity into trichloroacetic acid-precipitable protein was determined after 90 min of incubation (*shaded bars*). The average of values obtained from triplicate incubation samples is provided. Cell suspensions without labeled amino acid were also incubated for 90 min and growth hormone content was determined. Triplicate biological samples were prepared, and each was assayed for growth hormone in duplicate. Results are expressed as the increase in growth hormone ± SE (N = 6). Growth hormone at the start of the incubation period was 1.4 μg/mg of protein (*open bars*). Results provided in this figure were obtained from a single experiment. Findings have been reproduced in two separate experiments.

TABLE 5

Effects of inhibitors of leucine incorporation on production of growth hormone and prolactin by GH_3 cells

Ca^{2+}-depleted cells were suspended in buffered Joklik's MEM containing 1 mM EGTA, 1 mg/ml of bovine serum albumin, and the indicated concentrations of inhibitors and $CaCl_2$ and were incubated for 90 min. For each experimental value, triplicate biological samples were prepared, and each was assayed for hormone content in duplicate. Results are expressed as the increase in total growth hormone or prolactin (cells and extracellular medium) ± SE (N = 6). At the start of the incubation, growth hormone was 2.98 μg/mg of protein, and prolactin was 0.58 μg/mg of protein. Results of a single experiment are provided. Findings have been reproduced in two separate experiments.

Additive	0.9 mM Ca^{2+}		1.5 mM Ca^{2+}	
	Increase in growth hormone	Increase in prolactin	Increase in growth hormone	Increase in prolactin
	μg/mg of protein/90 min		μg/mg of protein/90 min	
None	1.30 ± 0.03	0.25 ± 0.01	1.95 ± 0.03	0.41 ± 0.01
Acetylcholine				
30 nM	1.15 ± 0.08	0.25 ± 0.01	nd[a]	nd
100 nM	0.88 ± 0.01	0.15 ± 0.01	nd	nd
1 μM	0.83 ± 0.07	0.16 ± 0.01	1.95 ± 0.15	0.38 ± 0.01
Trifluoperazine				
1 μM	1.07 ± 0.07	0.16 ± 0.02	nd	nd
3 μM	0.53 ± 0.07	0.12 ± 0.01	nd	nd
10 μM	0.20 ± 0.01	0.09 ± 0.01	1.78 ± 0.06	0.30 ± 0.01
20 μM	8.13 ± 0.03	0.06 ± 0.01	1.09 ± 0.09	0.28 ± 0.02
Chlorpromazine				
1 μM	1.25 ± 0.04	0.20 ± 0.01	nd	nd
3 μM	0.76 ± 0.02	0.12 ± 0.02	nd	nd
10 μM	0.18 ± 0.04	0.10 ± 0.01	1.95 ± 0.02	0.38 ± 0.01
Nifedipine				
1 μM	0.51 ± 0.05	0.17 ± 0.03	1.81 ± 0.19	0.38 ± 0.01

[a] nd, not determined.

for inhibition by chlorpromazine, trifluoperazine, ergotamine, and bromocriptine. Furthermore, submaximal combinations of nifedipine and chlorpromazine resulted in additive effects.[1] Since nifedipine and verapamil decrease both intracellular free Ca^{2+} concentrations and prolactin release in GH_4C_1 cells (36), and since both drugs inhibit leucine incorporation by a Ca^{2+}-dependent mechanism, inhibition of amino acid incorporation by prolactin secretion inhibitors presumably results from alterations in Ca^{2+} metabolism. A probable mechanism by which these agents affect protein synthesis in GH_3 cells is through inhibition of Ca^{2+} uptake. This hypothesis is explored in the accompanying paper (23).

The secretion of prolactin was not measured under the conditions used in this study. Consequently, it cannot be stated

definitively that inhibition of this process by the drugs involves a mechanism similar to that for inhibition of protein synthesis, although such a mechanism is consistent with currently available data. That these agents reduce both prolactin synthesis and secretion in a coordinate fashion under physiologic conditions remains to be demonstrated.

The extracellular free Ca^{2+} concentrations at which these drugs inhibit amino acid incorporation were not determined for the enriched medium used in this study. However, in experiments conducted in buffered saline, pH 7.5, lacking albumin and containing 1 mM EGTA, maximal leucine incorporation by GH_3 cells was observed at 1.2 mM added Ca^{2+} and full inhibition by 1 μM nifedipine at 1.1 mM but not at 1.2 mM added cation. It is likely, therefore, that nifedipine and other agents at the concentrations employed in this study inhibit protein synthesis by GH_3 cells only at free Ca^{2+} concentrations below 200 μM. Such free Ca^{2+} concentrations are considerably lower than those present under physiologic conditions. It should be noted, however, that these studies were conducted using tumor cells which are recognized to require considerably lower Ca^{2+} concentrations for growth and proliferation than do normal cell types (37). Additional work will be required to establish whether these drugs inhibit protein synthesis in normal cells of the anterior pituitary under conditions encountered *in vivo*.

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