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Mechanisms of Action of Inhibitors of Prolactin Secretion in GH_3 Pituitary Cells. II. Blockade of Voltage-Dependent Ca^{2+} Channels

SUSAN E. WOLFE and MARGARET A. BROSTROM

Department of Pharmacology, University of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway, New Jersey 08854

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SUMMARY

Pharmacologic agents reported to inhibit prolactin secretion by GH_3 pituitary cells were observed to inhibit protein synthesis by these cells in a Ca^{2+}-dependent manner. The possibility that these substances exert their effects on protein synthesis by restricting intracellular Ca^{2+} availability was explored. Trifluoperazine and chlorpromazine at concentrations that inhibit amino acid incorporation reduced uptake of $^{45}Ca^{2+}$ by intact cells approximately 30% under nondepolarizing conditions. Increased extracellular K^+ (30 mM), which depolarizes the membrane and opens the voltage-dependent Ca^{2+} channel of GH_3 cells, produced a 2-fold increase in $^{45}Ca^{2+}$ uptake; phenothiazines fully suppressed this effect of K^+. Nifedipine, verapamil, ergotamine, bromocriptine, (+)- and (−)-butaclamol, and calmidazolium were also effective in inhibiting $^{45}Ca^{2+}$ uptake under depolarizing and nondepolarizing conditions. The membrane potential of either depolarized or nondepolarized cells, as determined by [^{3}H]tetraphenylphosphonium$^+$ distribution, was not affected significantly by secretory inhibitors. Increased extracellular K^+ altered protein synthesis by GH_3 cells in a biphasic manner. Amino acid incorporation by cells incubated at low extracellular Ca^{2+} concentrations was stimulated by K^+, whereas incorporation by cells in high Ca^{2+} medium was inhibited by K^+. Trifluoperazine, chlorpromazine, nifedipine, and bromocriptine suppressed both effects of K^+ on protein synthesis. It is proposed that these antagonists of secretion inhibit protein synthesis by GH_3 cells through blockade of voltage-dependent Ca^{2+} channels.

An increase in intracellular free calcium concentration serves as a signal to promote various specialized cellular processes such as muscle contraction and secretion of neurotransmitters and hormones. Although millimolar concentrations of Ca^{2+} are present in extracellular fluids, the intracellular concentration of the free Ca^{2+} pool is maintained at approximately 0.1–0.3 μM in unstimulated cells through close regulation of Ca^{2+} influx, through active extrusion of the cation and through Na^+-Ca^{2+} exchange. Intracellular Ca^{2+} concentrations, however, may be increased in excess of an order of magnitude in response to electrical or chemical stimulation as a result of mobilization of intracellular Ca^{2+} stores and/or via entry of calcium from extracellular fluids. The voltage-dependent calcium channel represents an important regulatory pathway through which Ca^{2+} enters excitable cells.

Cloned GH cell lines are commonly employed model systems for investigation of the mechanisms involved in hormone release. Electrophysiological studies have demonstrated that GH_3 cells, like normal anterior pituitary cells, spontaneously generate action potentials and possess voltage-dependent Ca^{2+} channels (1, 2). The properties of these Ca^{2+} channels have been investigated using patch electrode voltage clamp (3) and ion flux techniques (4). The Ca^{2+} channel in GH cells is subject to blockade by the organic Ca^{2+} channel antagonists. Pharmacologic manipulation of the state of the voltage-dependent Ca^{2+} channel results in alterations in the rate of hormone secretion. Prolactin release is stimulated in response to depolarizing concentrations of extracellular K^+ which increase both the influx of Ca^{2+} through the voltage-dependent Ca^{2+} channel (4) and the free intracellular concentration of Ca^{2+} (5). Verapamil and nifedipine suppress basal and K^+-stimulated prolactin release (6–8).

Secretion of prolactin by anterior pituitary (9), but not by GH_3 cells (10), is inhibited by physiologic (nM) concentrations of dopamine agonists. It is presumed that GH_3 cells lack the high affinity dopamine receptors found in cells of the normal anterior pituitary (9). However, both GH_3 cells (11) and normal pituitary cells (9) have a lower affinity binding site, and secretion of prolactin by both cell types is suppressed by pharmacologic (μM) concentrations of dopaminergic agonists and antagonists including the phenothiazines trifluoperazine and chlorpromazine (10, 12, 13–15). Although the (+)-isomer of butaclamol typically binds to dopamine receptors with a 1000-fold greater affinity than does the (−)-isomer (9), the (+)- and

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ABBREVIATIONS: TPP, tetraphenyl phosphonium; MEM, modified Eagle's medium; EGTA, ethylene glycolbis (β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; DMSO, dimethylsulfoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(−)-isomers of butaclamol have similar affinities for this low affinity receptor (11) and are equally effective in inhibiting prolactin release (10). Therefore, inhibition of secretion by dopamine antagonists and agonists in GH_3 cells is most likely mediated through binding to this low affinity receptor. Phenothiazines, in contrast, have been proposed to inhibit prolactin release through an interaction with the Ca^{2+} binding protein, calmodulin (16). Evidence also exists suggesting that Ca^{2+} is involved in mediating responses to both dopamine antagonists and agonists. Trifluoperazine blocks calcium-dependent action potentials in GH_3 cells (16) and decreases high K^+-induced Ca^{2+} uptake in both rat synaptosomes (17) and anterior pituitary cells (18). Chlorpromazine and haloperidol inhibit Ca^{2+} influx and tension development in vascular smooth muscle (19). In the preceding paper (20), it was shown that protein synthesis is inhibited in a Ca^{2+}-dependent manner by antagonists of prolactin secretion in GH_3 cells. Leucine incorporation into protein was inhibited significantly by trifluoperazine, chlorpromazine, bromocriptine, ergotamine, and both (+)- and (−)-butaclamol. At high extracellular Ca^{2+} concentrations this inhibition was completely reversed. It was proposed that these inhibitors decreased amino acid incorporation by decreasing Ca^{2+} availability at key regulatory sites in the cell. Since nifedipine reduced leucine incorporation to the same extent and with the same dependence on extracellular Ca^{2+} concentration as other inhibitors of prolactin secretion, inhibition of Ca^{2+} influx was suggested as a possible mechanism through which such changes in Ca^{2+} availability could arise.

The present report describes the effects of secretory inhibitors on $^{45}Ca^{2+}$ uptake and leucine incorporation in depolarized and nondepolarized cells. Those agents observed to inhibit protein synthesis in a manner reversible by Ca^{2+} were found to decrease basal Ca^{2+} uptake as well as uptake in the presence of depolarizing concentrations of K^+. In addition, these secretory inhibitors reversed the effects of increased extracellular K^+ on amino acid incorporation. These results are consistent with the hypothesis that inhibition of protein synthesis by secretory antagonists occurs as a consequence of blockade of the voltage-dependent Ca^{2+} channel.

Experimental Procedures

Materials. Dinonyl phthalate, TPP bromide, $^{45}CaCl_2$ (17 mCi/mg), and L-[4,5-^{3}H]leucine (58 Ci/mmol) were purchased from International Chemical and Nuclear Corp., and silicon oil was purchased from Ace Scientific. [Phenyl-^{3}H]TPP bromide (35.5 ci/mmol) was obtained from New England Nuclear. Other reagents and drugs were obtained from the sources acknowledged in the preceding paper (20).

Cell culture and harvesting of GH_3 cells. Cells were cultured and harvested as described previously (20). Routinely, cells were washed twice by resuspension in buffered Ham's F-10 medium followed by centrifugation prior to use. Ca^{2+}-depleted cells were prepared by washing cells with Joklik's low Ca^{2+} MEM containing 1 mM EGTA as described (20).

Measurement of $^{45}Ca^{2+}$ uptake. Aliquots (0.5 ml, 1–2 × 10^6 cells) of washed GH_3 cells in buffered Ham's F-10 (0.3 mM Ca^{2+}) were pretreated with additives for 15 min at room temperature. Most additives were dissolved in buffered saline and added in 10-μl volumes. Bromocriptine, ergotamine, and nifedipine were prepared in DMSO and diluted in saline prior to addition such that the final concentration of DMSO did not exceed 0.1%, v/v. Addition of this concentration of DMSO in the absence of drugs did not effect $^{45}Ca^{2+}$ uptake. KCl (25 mM) was then added with 1 μCi of $^{45}Ca^{2+}$ and incubation continued at room temperature. Cells were separated from the medium by centrifugation of aliquots (0.4 ml) of the cell suspension through 200 μl of dinonyl phthalate/silicon oil (1:1 v/v) (21) for 30 sec in a Beckman Microfuge. The supernatant fluid was aspirated and the centrifuge tubes were inverted to drain. The tips of the tubes containing the pellets were then severed and placed in glass vials, and the pellets were solubilized in 500 μl of 1% sodium dodecyl sulfate. $^{45}Ca^{2+}$ in combination with 1 mM EGTA was added to an equivalent number of cells, and the preparations were immediately centrifuged through oil to assess the contribution of extracellular $^{45}Ca^{2+}$. 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 ± standard error of values obtained for triplicate samples.

Determination of membrane potential. Membrane potentials were ascertained from the steady state distribution of [^{3}H]TPP^+ between the extracellular and intracellular fluid (4). Aliquots of washed GH_3 cells were pretreated with agents of interest for 15 min at room temperature. [^{3}H]TPP^+(2 μM, 7 × 10^5 cpm/ml) was then added and incubation continued for 20 min. Cells were separated from extracellular fluids by centrifugation through oil, the tip of the centrifuge tube was severed, and the pellet was solubilized as described above. The contribution of extracellular [^{3}H]TPP^+ to the cell pellets was estimated by adding 50 μM unlabeled TPP^+ to an equivalent number of cells in combination with [^{3}H]TPP^+ followed immediately by centrifugation of the suspension through oil. This blank value was subtracted from all experimental values.

Miscellaneous procedures. Methods for determining protein concentration and [^{3}H]leucine incorporation into protein were as described in the preceding paper (20).

Results

Inhibition of $^{45}Ca^{2+}$ uptake by selected inhibitors of amino acid incorporation. $^{45}Ca^{2+}$ uptake into GH_3 cells was measured at various times following suspension of cells in Ham's F-10 medium containing 0.3 mM Ca^{2+} (Fig. 1). Uptake of $^{45}Ca^{2+}$ was increased 2-fold by raising extracellular K^+ to 30 mM, a concentration reported to induce membrane depolarization and activation of Ca^{2+} influx via voltage-dependent Ca^{2+} channels (4). The phenothiazine, chlorpromazine, inhibited basal uptake of $^{45}Ca^{2+}$ by 30% and completely reversed the effect of high K^+ on $^{45}Ca^{2+}$ uptake. Trifluoperazine blocked

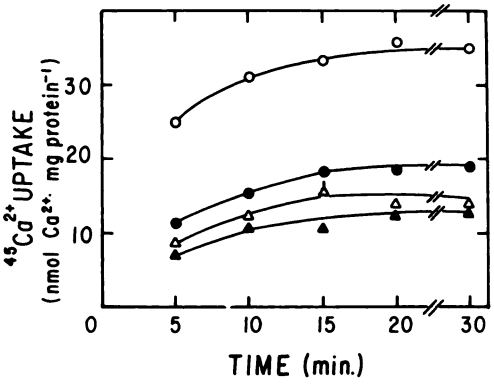

Fig. 1. Effect of chlorpromazine on basal and high K^+-stimulated $^{45}Ca^{2+}$ uptake by GH_3 cells. GH_3 cells were washed twice with Ham's F-10 medium and resuspended in Ham's F-10 medium containing 25 mM Hepes, pH 7.4, and 1 mg/ml of fatty acid-free bovine serum albumin without (●, ○) or with (▲, △) 10 μM chlorpromazine. KCl at 25 mM (○, △) was added to half of the preparations. $^{45}Ca^{2+}$ (2 μCi/ml of cell suspension) was then added to all preparations, and at the indicated times 300-μl aliquots were withdrawn for measurement of cell-associated $^{45}Ca^{2+}$. Values shown are the means ± SE of values obtained from triplicate incubation samples for a single experiment. Findings have been reproduced in two separate experiments.

basal and K^+ stimulated uptake to the same extent as did chlorpromazine (data not shown).

The abilities of the ergot alkaloids, bromocriptine and ergotamine, the dopaminergic antagonist, (+)-butaclamol and its inactive isomer, (−)-butaclamol, and the putative calmodulin antagonist, calmidazolium, to block $^{45}Ca^{2+}$ uptake were tested at both physiologic and high concentrations of K^+ (Table 1). Both basal and K^+-stimulated $^{45}Ca^{2+}$ uptake were inhibited by each of these drugs, and the extents of inhibition were similar to those obtained with maximally effective concentrations of the Ca^{2+} channel antagonists, nifedipine and verapamil.

Prolactin secretion (22, 23) and amino acid incorporation (2) are inhibited by muscarinic agonists. However, neither acetylcholine nor carbachol had a significant effect on either basal uptake or high K^+-stimulated uptake of $^{45}Ca^{2+}$ (Table 1), indicating that inhibition by muscarinic agonists is mediated by a different mechanism from that observed for the other secretory inhibitors.

The concentration dependence of chlorpromazine (Fig. 2A) and trifluoperazine (Fig. 2B) producing inhibition of $^{45}Ca^{2+}$ uptake into cells incubated at physiological or high K^+ concentrations was investigated. Chlorpromazine (1 μM) significantly decreased basal and high K^+-stimulated uptake. Basal uptake of $^{45}Ca^{2+}$ was decreased 40% by a 20 μM drug concentration. High K^+ increased $^{45}Ca^{2+}$ uptake by 80%, and this effect was completely reversed in the presence of 10 μM chlorpromazine. At this concentration of phenothiazine, $^{45}Ca^{2+}$ uptake was lower than that obtained in the absence of drug under basal conditions. In contrast, chlorpromazine sulfoxide, an analog of chlorpromazine lacking dopamine binding (24) and calmodulin antagonist (16) activities, at a concentration of 20 μM had no effect on basal $^{45}Ca^{2+}$ uptake and reduced high K^+-stimulated uptake by less than 10%. Trifluoperazine was as effective as but slightly less potent than chlorpromazine. Trifluoperazine (1 μM) did not inhibit $^{45}Ca^{2+}$ uptake, but a 10 μM drug concentration decreased uptake to the same extent as 10 μM chlorpromazine in the absence or presence of elevated K^+.

TABLE 1

Effects of selected agents on basal and high K^+-stimulated $^{45}Ca^{2+}$ uptake in GH_3 cells

Cells were pretreated with the indicated concentrations of additives for 15 min in buffered Ham's F-10 medium containing 1 mg/ml of bovine serum albumin. Portions of the samples were treated with 25 mM KCl. $^{45}Ca^{2+}$ (2 μCi/ml of cell suspension) was then added, and cell-associated $^{45}Ca^{2+}$ was measured after 15 min of incubation. Results are expressed as the average ± SE ($N = 3$) of values obtained for a single experiment. Findings have been reproduced in three separate experiments.

Additive		$^{45}Ca^{2+}$ uptake (nmol of Ca^{2+}/mg of protein/15 min)	
		Control	+25 mM KCl
None		22.1 ± 0.3	34.0 ± 0.8
Bromocriptine	(1 μM)	17.7 ± 0.2	28.0 ± 0.8
	(3 μM)	16.7 ± 0.1	24.4 ± 0.4
	(10 μM)	16.0 ± 0.4	19.0 ± 0.5
Ergotamine	(1 μM)	21.4 ± 0.5	29.8 ± 0.6
	(3 μM)	19.5 ± 1.0	26.1 ± 0.6
	(10 μM)	16.8 ± 0.8	18.2 ± 0.9
Calmidazolium	(1 μM)	19.9 ± 0.4	22.7 ± 0.6
(+)-Butaclamol	(10 μM)	17.2 ± 0.1	24.5 ± 0.7
(−)-Butaclamol	(10 μM)	16.3 ± 0.2	18.8 ± 0.7
Nifedipine	(0.1 μM)	20.2 ± 0.1	22.5 ± 0.4
	(1 μM)	17.8 ± 0.8	19.3 ± 0.3
Verapamil	(10 μM)	20.6 ± 0.4	18.5 ± 0.7
Acetylcholine	(1 μM)	22.6 ± 0.2	35.9 ± 1.0
Carbachol	(10 μM)	22.2 ± 0.5	36.0 ± 0.5

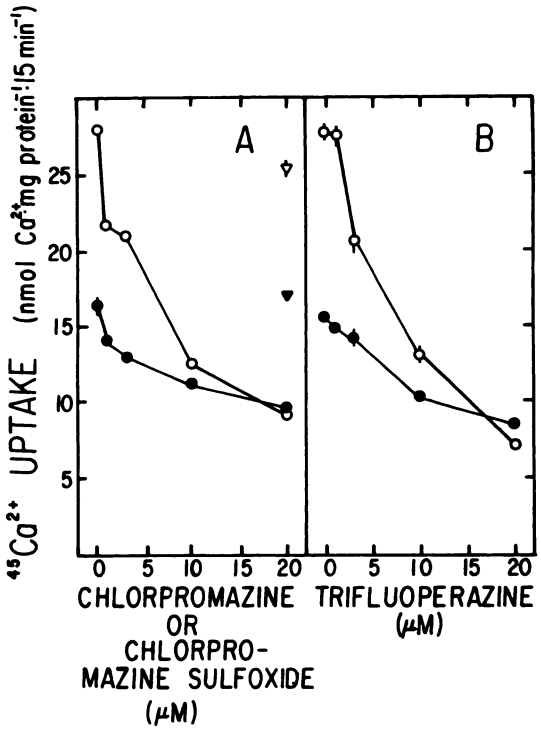

Fig. 2. Chlorpromazine, chlorpromazine sulfoxide, and trifluoperazine concentration dependencies of inhibition of basal and high K^+-stimulated $^{45}Ca^{2+}$ uptake by GH_3 cells. Cells were pretreated with the indicated concentrations of (A) chlorpromazine (●, ○) or chlorpromazine sulfoxide (▼, ▽), or (B) trifluoperazine for 15 min in Ham's F-10 medium containing 25 mM Hepes, pH 7.4, and 1 mg/ml of bovine serum albumin. Portions of samples were then treated with 25 mM KCl (○, ▽), and $^{45}Ca^{2+}$ (2 μCi/ml of cell suspension) was added to all samples. Measurement of cell-associated $^{45}Ca^{2+}$ was performed after 15 min of incubation. Each *point* represents the mean value ± SE of triplicate incubation samples for a single experiment. Findings have been reproduced in two separate experiments.

Effect of increased extracellular K^+ on amino acid incorporation. The effect of depolarizing concentrations of K^+ on leucine incorporation by GH_3 cells was examined (Fig. 3). Cells were Ca^{2+}-depleted by successive washes with Joklik's MEM adjusted to 1 mM EGTA. KCl (25 mM) was added to portions of the cell suspensions, and all preparations were treated with various concentrations of Ca^{2+}. Incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material was determined after 60 min of incubation. In control preparations, amino acid incorporation was inhibited at added Ca^{2+} concentrations below 1.0 mM. High K^+ either stimulated or inhibited amino acid incorporation depending on the concentration of extracellular Ca^{2+}. Leucine incorporation was increased above control values when cell preparations were exposed to less than 0.9 mM added Ca^{2+}. However, at higher concentrations of Ca^{2+}, amino acid incorporation by high K^+-treated cells was inhibited; at 1.05 mM or more added Ca^{2+}, leucine incorporation was half the rate observed for control cultures. Maximal rates of incorporation observed for control preparations were never attained in cells exposed to high K^+. Effects of high K^+ on leucine incorporation were not due to increased osmolarity or chloride concentration of the medium, since increasing the concentration of NaCl by 25 mM did not affect the extent of amino acid incorporation at any concentration of Ca^{2+} tested (data not shown).

Reversal of effects of K^+ on amino acid incorporation.

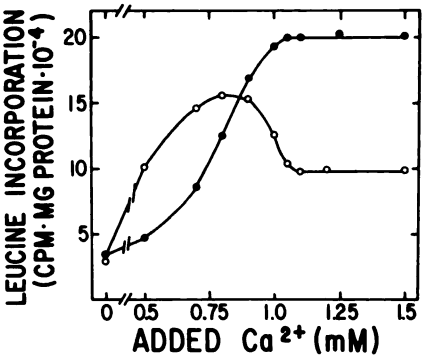

Fig. 3. Effects of increased extracellular K^+ concentrations on the Ca^{2+} concentration dependence of amino acid incorporation by GH_3 cells. Ca^{2+}-depleted cells were suspended in Joklik's MEM, pH 7.4, containing 25 mM Hepes, 1 mM EGTA, and 1 mg/ml of fatty acid-free bovine serum albumin. Cell suspensions were pretreated for 5 min with no added KCl (●) or 25 mM KCl (○) and with the indicated added concentrations of $CaCl_2$. [^{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 60 min of incubation. The mean value of triplicate incubation samples is provided for a single experiment. Results have been reproduced in four separate experiments.

TABLE 2

Effects of variations in extracellular K^+ and Ca^{2+} concentrations on amino acid incorporation in the absence and presence of chlorpromazine

Ca^{2+}-depleted cells were incubated in Hepes-buffered saline, pH 7.4, containing 1 mM $MgCl_2$, 1 mM EGTA, 20 μM leucine, 1 mg/ml of glucose, 1 mg/ml of bovine serum albumin, and the indicated concentrations of $CaCl_2$ and K^+. The monovalent cation concentration of all samples was adjusted with Na^+ to 145 mM. Chlorpromazine (cpz) (10 μM) was added to portions of the cell suspensions. Following a 10-min pretreatment, [^{3}H]leucine (5×10^6 cpm/0.5 ml of cell suspension) was added to all samples and incorporation of radioactivity into trichloroacetic acid-insoluble protein was determined after 30 min of incubation. Values obtained from duplicate incubation samples are provided for a single experiment. Findings have been reproduced in three separate experiments.

Added Ca^{2+}	Additive	Leucine incorporation (cpm × 10^{-4}/mg of protein)		
		5 mM K^+	15 mM K^+	25 mM K^+
0.5 mM		7.5, 7.7	11.0, 10.5	13.6, 16.5
	cpz	5.4, 5.5	5.9, 6.3	5.8, 6.2
0.8 mM		12.0, 12.7	36.9, 38.6	49.5, 47.7
	cpz	5.7, 6.1	6.5, 6.3	6.2, 6.6
1.0 mM		48.6, 37.6	52.3, 50.7	47.4, 46.2
	cpz	6.6, 6.7	6.9, 8.5	10.4, 14.8
1.5 mM		52.4, 53.1	43.2, 43.0	35.5, 36.1
	cpz	52.7, 52.0	49.5, 50.2	51.1, 49.0

The ability of chlorpromazine to reverse the effects of increased extracellular K^+ on leucine incorporation by GH_3 cells was investigated (Table 2). Chlorpromazine, as described in the preceding paper (20), increased the extracellular Ca^{2+} concentration required for maximal rates of protein synthesis. Addition of 15 or 25 mM K^+ to the medium resulted in increased leucine incorporation at low extracellular Ca^{2+} (0.5 or 0.8 mM added Ca^{2+}) and decreased amino acid incorporation at higher concentrations of Ca^{2+} (1.5 mM added Ca^{2+}). In the presence of chlorpromazine, however, both effects of K^+ on leucine incorporation were overturned and, as was observed at physiological K^+ concentrations, amino acid incorporation was inhibited at limiting Ca^{2+} concentrations. Inhibition of leucine incorporation by chlorpromazine was partially overcome by addition of 25 mM K^+ to the medium when Ca^{2+} concentrations were restored to 1.0 mM extracellular cation. The abilities of bromocriptine, trifluoperazine, and nifedipine to reverse the effects of high extracellular K^+ on leucine incorporation were also examined for cells treated with either 0.5 mM, 0.9 mM, or 1.5 mM $CaCl_2$ (Table 3). Each agent reversed the stimulatory effect of high K^+ seen at low concentrations of extracellular Ca^{2+} as well as the inhibitory effect observed at higher extracellular Ca^{2+} concentrations. These results are consistent with K^+ stimulation of leucine incorporation via increased Ca^{2+} uptake and with inhibition of amino acid incorporation by drugs through blockade of Ca^{2+} uptake. Acetylcholine, which had no effect on $^{45}Ca^{2+}$ uptake, similarly did not antagonize either effect of high K^+ on leucine incorporation.

It was desirable to correlate Ca^{2+} uptake with amino acid incorporation in the absence or presence of high K^+ and/or drugs. Since stimulation of amino acid incorporation occurred at low extracellular Ca^{2+} concentrations, it was necessary to employ a Ca^{2+}-EGTA-buffered system to observe this stimulation. For studies of $^{45}Ca^{2+}$ uptake, however, this approach was not technically feasible. However, amino acid incorporation was inhibited at high K^+ concentrations in the absence of chelator and this inhibition was overturned by chlorpromazine and bromocriptine (data not shown). To test the possibility that the inhibitory effects of high K^+ on protein synthesis were mediated by Ca^{2+} entry through voltage-dependent Ca^{2+} channels, [^{3}H]leucine incorporation and $^{45}Ca^{2+}$ uptake were measured for a single preparation of non-Ca^{2+}-depleted GH_3 cells as a function of extracellular K^+ at Ca^{2+} concentrations thought to be present in extracellular fluids (Fig. 4). $^{45}Ca^{2+}$ uptake was found to be dependent on both K^+ and Ca^{2+}. By contrast, amino acid incorporation was inhibited as the concentration of extracellular K^+ was increased, but this inhibition was independent of the extracellular Ca^{2+} concentration. Chlorpromazine blocked the increase in $^{45}Ca^{2+}$ uptake at high K^+, but the drug was less effective in reversing inhibition of leucine incorporation at high K^+. It appeared, therefore, that Ca^{2+} did not directly mediate the inhibition of amino acid incorporation by high K^+.

Effects of inhibitors on K^+-induced membrane depolarization. The observed blockade by these agents of the effects of depolarizing concentrations of K^+ on $^{45}Ca^{2+}$ uptake and [^{3}H]leucine incorporation could have conceivably arisen as a result of nonspecific membrane stabilization with resultant prevention of depolarization in response to elevated K^+. Each of these drugs is lipophilic and has been reported to stabilize membranes (25), albeit at higher concentrations than those used in these experiments. Therefore, the steady state distribution of the lipophilic cation [^{3}H]TPP^+, which is commonly used as a measure of membrane potential, was determined for depolarized and nondepolarized cells treated with chlorpromazine, trifluoperazine, bromocriptine, ergotamine, or nifedipine (Table 4). Cells were pretreated with inhibitors in medium containing either 5 mM or 30 mM K^+, and [^{3}H]TPP^+ uptake was measured after 20 min when equilibrium conditions prevailed (4). Bromocriptine, trifluoperazine, and ergotamine increased [^{3}H]TPP accumulation slightly under nondepolarizing conditions, whereas chlorpromazine and nifedipine did not change [^{3}H]TPP^+ accumulation significantly. Addition of 25 mM K^+ to GH_3 cells decreased [^{3}H]TPP^+ accumulation 55% in the absence of drugs and 40–50% in the presence of chlorpromazine, trifluoperazine, ergotamine, or bromocriptine. The minimal effects of these drugs on membrane depolarization, therefore, could not account for their actions in reversing the effects of K^+ on amino acid incorporation or Ca^{2+} uptake.

TABLE 3

Antagonism of effects of increased extracellular K^+ on leucine incorporation by inhibitors of Ca^{2+} uptake

Ca^{2+}-depleted cells were suspended in buffered Joklik's MEM containing 1 mM EGTA and 1 mg/ml of fatty acid-free bovine serum albumin. Cells were pretreated for 15 min with the indicated concentrations of $CaCl_2$ and additives. KCl (25 mM) was then added to half of the preparations. Following addition of [^{3}H]leucine (2×10^6 cpm/0.5 ml of cell suspension), all samples were incubated for 90 min and trichloroacetic acid-insoluble radioactivity was determined. Results are expressed as the average of values obtained for triplicate incubation samples from a single experiment. Findings have been reproduced in two separate experiments.

Additive		Leucine incorporation ($cpm \times 10^{-3}$/mg of protein/90 min)					
		Control			+25 mM KCl		
		0.5 mM $CaCl_2$	0.9 mM $CaCl_2$	1.5 mM $CaCl_2$	0.5 mM $CaCl_2$	0.9 mM $CaCl_2$	1.5 mM $CaCl_2$
None		36.2	265	273	69.1	243	190
Chlorpromazine	(3 μM)	25.2	29.1	271	22.3	38.1	241
	(10 μM)	20.9	22.8	274	19.9	20.4	271
Trifluoperazine	(10 μM)	21.0	22.4	283	23.0	21.6	234
Bromocriptine	(10 μM)	23.7	27.3	291	25.3	29.2	236
Nifedipine	(1 μM)	24.1	58.2	280	30.0	99.9	226
Acetylcholine	(1 μM)	28.7	128	272	63.9	239	194

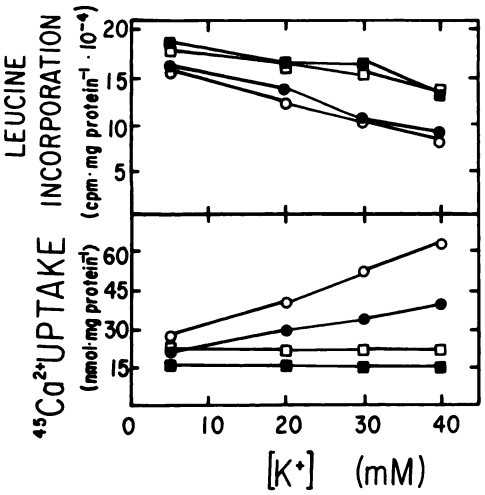

Fig. 4. Effects of varying K^+ concentrations on [^{3}H]leucine incorporation and $^{45}Ca^{2+}$ uptake in the presence and absence of chlorpromazine. Non-Ca^{2+}-depleted GH_3 cells were suspended in Hepes-buffered saline, pH 7.4, lacking EGTA and containing 1 mM $MgCl_2$, 1 mg/ml of glucose, 1 mg/ml of bovine serum albumin, 20 μM leucine, and either 0.5 mM (●, ■) or 1.0 mM (○, □) $CaCl_2$. The monovalent cation concentration of all samples was adjusted with Na^+ to 145 mM. Cells were pretreated without (○, ●) or with (□, ■) 10 μM chlorpromazine for 10 min. $^{45}Ca^{2+}$ (1 μCi/ml of cell suspension or [^{3}H]leucine (5×10^5 cpm/ml of cell suspension) was then added to appropriate samples. $^{45}Ca^{2+}$ uptake was measured following 15 min of incubation and incorporation of [^{3}H-leucine into trichloroacetic acid-insoluble protein after 30 min of incubation. The mean of values obtained from triplicate incubation samples for measurements of $^{45}Ca^{2+}$ uptake and for measurements of leucine incorporation are provided for a single experiment. Findings have been reproduced in two separate experiments.

Discussion

Selected agents that inhibit prolactin secretion by GH_3 cells were observed to inhibit amino acid incorporation in these cells at limiting Ca^{2+} concentrations (20). In the presence of the dopamine agonists, ergotamine or bromocriptine, or the phenothiazine dopaminergic antagonists, trifluoperazine or chlorpromazine, the Ca^{2+} concentration dependence of amino acid incorporation was altered such that higher concentrations of extracellular Ca^{2+} were required. The voltage-dependent Ca^{2+} channel antagonists, nifedipine and verapamil, inhibited amino acid incorporation to the same extent and with the same Ca^{2+} concentration dependence as the dopamine agonists and antagonists. Therefore, inhibition of Ca^{2+} uptake through specialized

TABLE 4

Effect of inhibitors of Ca^{2+} uptake on [^{3}H]TPP^+ accumulation by GH_3 cells in medium containing 5 mM or 30 mM K^+

GH_3 cells were incubated in buffered saline containing 25 mM Hepes, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mg/ml of glucose, and either 5 mM or 30 mM K^+. The concentration of choline$^+$ was adjusted such that the monovalent cation concentration was 145 mM. Following a 15-min pretreatment period with the indicated concentrations of inhibitors, 2 μM [^{3}H]TPP^+ (7×10^5 cpm/ml of cell suspension) was added, and uptake of radioactivity was determined after 20 min of incubation. Values shown are the means ± SE ($N = 4$) for a single experiment. Findings have been reproduced in two separate experiments.

Additive		[^{3}H]TPP^+ accumulation ($cpm \times 10^{-4}$/mg of protein/20 min)	
		5 mM K^+	30 mM K^+
None		28.1 ± 0.3	12.5 ± 0.1
Chlorpromazine	(3 μM)	27.4 ± 0.3	14.1 ± 0.1
	(10 μM)	27.6 ± 0.1	14.5 ± 0.2
Trifluoperazine	(10 μM)	32.1 ± 0.2	14.9 ± 0.3
Bromocriptine	(10 μM)	36.2 ± 0.3	17.5 ± 0.2
Ergotamine	(10 μM)	33.5 ± 0.4	16.4 ± 0.1
Nifedipine	(1 μM)	25.1 ± 0.3	12.1 ± 0.1

Ca^{2+} channels was proposed as a likely mechanism through which these agents inhibit protein synthesis in GH_3 cells (20). The results obtained in this study provide strong support for this hypothesis. Bromocriptine, ergotamine, chlorpromazine, and trifluoperazine each were found to inhibit significantly both basal and K^+-stimulated uptake of $^{45}Ca^{2+}$ by GH_3 cells. The efficacies of these four agents as inhibitors of Ca^{2+} uptake were comparable to those of nifedipine and verapamil. In addition, inhibition of $^{45}Ca^{2+}$ uptake by trifluoperazine and chlorpromazine was obtained at the same drug concentrations required for inhibition of protein synthesis. Finally, chlorpromazine sulfoxide, an inactive analog of chlorpromazine, did not influence either amino acid incorporation or Ca^{2+} uptake.

Each of the inhibitors used in this study is known to be lipophilic and to possess membrane-stabilizing properties (25). Inhibition of the effects of high K^+ on $^{45}Ca^{2+}$ uptake could therefore have been due to nonspecific membrane stabilization against depolarization. However, since chlorpromazine, trifluoperazine, bromocriptine, and ergotamine did not reverse the effect of 30 mM K^+ on [^{3}H]TPP^+ accumulation, this possibility was excluded.

It is unclear whether the inhibitors used in this study interact directly with Ca^{2+} channels, with specific receptors for neurotransmitters, or with uncharacterized sites on the membrane. Low affinity saturable binding sites for dopaminergic ligands

which lack the stereospecificity and selectivity typical of classic dopaminergic receptors are present on GH_3 cells (11). Binding of [^{3}H]spiroperidol to this site is both heat labile and pronase sensitive (11), suggesting that the receptor is a protein. The concentrations at which dopaminergic agonists or antagonists displace [^{3}H]spiperone bound to this receptor correlate well with the concentrations at which these agents are effective inhibitors of prolactin secretion (11), amino acid incorporation (20), and $^{45}Ca^{2+}$ uptake. Therefore, binding of the various agents at pharmacologic concentrations to this site may explain their observed actions. Conversely, trifluoperazine and other neurologic drugs inhibit the binding of [^{3}H]nitrendipine to rat cortical membranes (26). Conceivably, pharmacologic inhibition of Ca^{2+} uptake by GH_3 cells could occur as a consequence of drug binding at the voltage-dependent Ca^{2+} channel. A third possibility is that the low affinity receptor is associated with or coupled to the voltage-dependent Ca^{2+} channel.

Muscarinic agonists, like other classes of agents that inhibit prolactin secretion, were found to reduce amino acid incorporation in a Ca^{2+}-dependent fashion. Unlike the other secretory inhibitors examined, however, muscarinic agonists did not block Ca^{2+} uptake nor did they antagonize the effects of high K^+ on Ca^{2+} uptake and on amino acid incorporation. Further study will be needed to ascertain whether mechanisms such as those associated with membrane hyperpolarization or K^+ channel activation mediate the effects of muscarinic agonists on GH_3 cells.

Stimulation by K^+ of amino acid incorporation was observed to depend on the extracellular Ca^{2+} concentration and could therefore be explained on the basis of K^+-induced Ca^{2+} uptake. In contrast, inhibition by K^+ of amino acid incorporation was dependent on the extracellular K^+ concentration but not the extracellular concentration of Ca^{2+}. Since uptake of $^{45}Ca^{2+}$ increased as a function of the concentrations of both K^+ and Ca^{2+}, no direct correlation could be found between K^+-induced Ca^{2+} uptake and inhibition of protein synthesis. Alternatively, inhibition of amino acid incorporation by high K^+ could be mediated through increased uptake of Na^+ via the voltage-dependent Na^+ channel. In support of this hypothesis, nifedipine has been shown to block Na^+ channels as well as Ca^{2+} channels (27). Furthermore, elevated Na^+ concentrations are known to inhibit protein synthesis in cell-free systems.

Prolactin production by GH_3 cells was inhibited by high K^+ to the same extent as amino acid incorporation in this study although total cellular protein content was not affected.[1] A similar observation has been made with regard to acetylcholine biosynthesis and total protein concentration in depolarized muscle. The rate of synthesis of acetylcholine receptors in skeletal muscle cells both in tissue culture and *in vivo* is influenced by muscle activity. Membrane excitation via electrical stimulation or through depolarization by veratridine or in response to high K^+ results in a specific decrease in *de novo* receptor synthesis (28). In contrast, calcium channel antagonists increase receptor synthesis (29). Furthermore, acetylcholine receptor synthesis in cultured chick myotubes is reported to increase following a 2-day treatment with trifluoperazine or chlorpromazine but not with their sulfoxide derivatives (30). Net protein synthesis, however, determined over a two day incubation period with labeled amino acid was not affected by high K^+ (29, 30). Conceivably, depolarization by K^+ may decrease amino acid incorporation into all cellular proteins but affect only the net accumulation of certain proteins such as those destined for membrane insertion or secretion. For other polypeptides, changes in synthetic rates are anticipated to be counterbalanced by compensatory changes in rates of degradation. Through such a mechanism the synthesis of particular proteins such as prolactin or the acetylcholine receptor could be specifically "down regulated" in excitable cells.

Phenothiazines are commonly used as pharmacological probes for investigation of calmodulin-dependent processes. Trifluoperazine, chlorpromazine, and calmidazolium inhibit calmodulin-dependent enzymes at micromolar concentrations *in vitro*; consequently it is often assumed that inhibition of a cellular function following addition of these drugs proves the involvement of calmodulin in that function. However, it is clear that these reputed calmodulin antagonists also inhibit effectively both basal and high K^+-stimulated uptake of Ca^{2+}. Furthermore, the sulfoxide derivative of chlorpromazine, which lacks calmodulin antagonist activity (16) and is commonly used as a negative control for demonstration of calmodulin effects, also lacks the ability to antagonize Ca^{2+} uptake. Therefore, those studies reporting or proposing a calmodulin requirement for a given cellular process on the basis of phenothiazine inhibition of that process are subject to alternate interpretation.

[1] S. E. Wolfe, unpublished observation.

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Send reprint requests to: Dr. Margaret A. Brostrom, Department of Pharmacology, UMDNJ-Rutgers Medical School, Box 101, Piscataway, NJ.