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ROLE OF CALCIUM IN THE THYROTROPIN-RELEASING HORMONE-STIMULATED RELEASE OF PROLACTIN FROM PITUITARY CELLS IN CULTURE

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SUMMARY

Thyrotropin-releasing hormone (TRH) or 50 mM K^+ stimulated the acute release of prolactin from the GH_4C_1 strain of rat pituitary cells in culture. The enhanced release of prolactin was inhibited in a dose-related manner by the Ca^{+2} antagonist Co^{+2} (2.0 to 0.5 mM) as well as by the Ca^{+2} chelator EGTA (1.0 mM). Co^{+2} also reduced spontaneous basal prolactin release. There was partial reversal of the inhibitory effect of Co^{+2} (2.0 mM) by Ca^{+2} (2.0 mM) and complete reversal of the inhibitory effect of EGTA (1.0 mM) by Ca^{+2} (2.0 mM). The enhanced release of prolactin stimulated by 50 mM K^+ was maximal by 10-20 minutes in medium containing 0.67 to 0.74 mM Ca^{+2}. Na^+ (50 mM) did not mimic the effect of high K^+. We conclude that Ca^{+2} is an essential cation in mediating the actions of high external K^+ and TRH on the release of prolactin by GH_4C_1 cells.

INTRODUCTION

The importance of extracellular Ca^{+2} in stimulus-elicited secretion of protein hormones is widely recognized (1,2). There is less certainty about the role of external calcium in basal hormone release. In the specific case of prolactin- and thyrotropin-secreting cells of the anterior pituitary gland, recent electrophysiologic experiments have shown that these cells can generate calcium action potentials (3-5), which may be enhanced in frequency by the hypothalamic tripeptide thyrotropin-releasing hormone (3,5). Because this tripeptide stimulates the release of both prolactin and thyrotropin from their respective cell types (6), the possible role of extracellular Ca^{+2} in the action of thyrotropin-releasing hormone (TRH) as a secretagogue is of physiologic interest. Early experiments with hypothalamic extracts contain-

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ing TRH activity indicated that the stimulated release of thyrotropin from pituitary tissue *in vitro* depended on calcium in the external medium (7,8). In more recent experiments with prolactin-secreting GH_3 cells, Vale *et al.* (9) have reported that TRH produced a small, transient acceleration of the rate of ^{45}Ca efflux from the cells previously incubated with the tracer. Our previous studies on the role of calcium in the secretion and synthesis of prolactin and growth hormone by GH_3 cells did not examine the short-term hormone-releasing actions of TRH (10,11).

In this report we have investigated the role of Ca^{+2} in the TRH-mediated release of prolactin from the GH_4C_1 strain of rat pituitary cells. We have used high extracellular K^+ which depolarizes endocrine cells and enhances membrane permeability to Ca^{+2} (1), as well as the calcium chelating agent EGTA and the competititive calcium antagonist Co^{+2} (12,13).

METHODS AND MATERIALS

Cell culture. All experiments described in this report were performed with the GH_4C_1 cell strain which synthesizes prolactin but not thyrotropin (14). Methods of cell culture have been described (15). In brief, cells were grown in 60 mm plastic dishes at 37°C in Ham's F 10 medium supplemented with 15% horse serum and 2.5% fetal calf serum (designated F 10(+)). As described below, the acute (20 min) release of prolactin was measured in two media in addition to F 10(+). These media were Eagle's MEM for suspension culture supplemented with 15% horse serum and 2.5% fetal calf serum (designated Spinner), and Ham's F 10 Nutrient Mixture lacking serum and supplemented with lactalbumin hydrolysate (5 g/1000 ml) (designated F 10(-) + 1h). The potassium concentrations of these media ranged from 4.5 to 5.8 mM. The concentrations of total calcium, measured by fluorometric titration with a Corning model 940 calcium analyzer, in F 10(+), Spinner and F 10(-) + 1h media were 0.74, 0.47, and 0.67 mM, respectively. Cell protein was determined by the method of Lowry *et al.* (16) using bovine serum albumin as standard. In the experiments presented, none of the treatments used altered significantly the total cell protein per dish.

Measurement of prolactin release. Before starting a release experiment, the F 10(+) medium in which the cells had been grown was removed, the dishes were washed once with 3 ml of warm sterile saline and all dishes incubated at 37°C for 2 h in 3.0 ml of Spinner medium. At the end of the 2-h incubation, the Spinner medium was removed, and 2.0 ml of the appropriate fresh, pre-equilibrated (37°C, pH 7.4) test medium was added to each dish. The various treatments were then added immediately in a small volume (100 µl or less) from a 20- to 30-fold concentrated stock solution of the appropriate compound in homologous medium. This point was designated zero time and the cultures were returned to the humidified incubator (37°C, 5% CO_2, 95% air) for the times indicated in each experiment, usually 20 minutes. Care was taken to work as rapidly as possible with pre-equilibrated solutions in order

Table 1

EFFECTS OF K^+, Co^{++} AND TRH ON THE RELEASE OF PROLACTIN

Treatment	Prolactin in medium (ng/ml)*	
	Spinner	F 10(+)
None	500 ± 15	280 ± 20
TRH (100 nM)	900 ± 60	480 ± 30
KCl (50 mM)	980 ± 20	620 ± 20
$CoCl_2$ (2.0 mM)	325 ± 4	140 ± 0
$CoCl_2$ (1.0 mM)	380 ± 45	190 ± 20
$CoCl_2$ (0.5 mM)	385 ± 15	280 ± 10
KCl (50) + $CoCl_2$ (2.0)	400 ± 10	210 ± 10
KCl (50) + $CoCl_2$ (1.0)	770 ± 8	330 ± 10
KCl (50) + $CoCl_2$ (0.5)	865 ± 5	490 ± 20
TRH (100) + $CoCl_2$ (2.0)	535 ± 80	170 ± 0
TRH (100) + $CoCl_2$ (1.0)	695 ± 20	320 ± 10
TRH (100) + $CoCl_2$ (0.5)	690 ± 10	480 ± 10

*Mean values from duplicate culture dishes ± range. The values given are for prolactin accumulation in medium in 20 min. The mean protein concentrations per dish for cells in Spinner medium and F 10 (+) were 0.65 and 0.71 mg, respectively.

to minimize any possible effects of temperature or pH on prolactin release. At the end of the incubation period, medium was removed and stored at -20°C until it was assayed for prolactin content by microcomplement fixation immunoassay (17). We have shown previously that accumulation of prolactin in culture medium for periods of less than one hour represents secretion of previously synthesized stored hormone (18); we term this short-term process prolactin release to differentiate it from longer-term effects of agents such as TRH on the synthesis of new prolactin molecules (19).

Materials. Thyrotropin-releasing hormone (TRH) was the synthetic tripeptide from Abbott Laboratories. KCl, $CoCl_2$, $CaCl_2$ and ethyleneglycol bis(β-amino-ethyl-ether)-N,N'-tetraacetic acid (EGTA) were reagent grade chemicals. EGTA was prepared as the neutralized sodium salt but designated simply as EGTA rather than NaEGTA.

RESULTS

When TRH or high K^+ were added to cultures of GH_4C_1 cells in either Spinner or F 10(+) medium, there was enhancement of the release of prolactin (Table 1). $CoCl_2$, at concentrations of 1.0 and 2.0 mM, inhibited the spontaneous release of prolactin, and also, in a dose-related manner (2.0 to 0.5 mM), $CoCl_2$ antagonized prolactin release stimulated by both 50 mM KCl and TRH (Table 1).

Because partial inhibition of TRH- and K^+-enhanced release was still detected at 0.5 mM $CoCl_2$, additional experiments were performed at lower cobalt concentrations. Again $CoCl_2$ (2.0 and 0.67 mM) inhibited spontaneous PRL release as well as the enhanced release induced by high K^+ and TRH (Table 2). However, these antagonistic effects of $CoCl_2$ on both spontaneous and stimulated release were not observed at 0.22 mM $CoCl_2$ (Table 2).

The time-course and ion specificity of the enhancement of prolactin release by high K^+ were examined. In F 10(+) and F 10(-) + 1h media, added K^+ stimulated the release of prolactin maximally by 20 and 10 minutes, respectively (Fig. 1). The effect in F 10(+) medium could have been maximal by 10 minutes; no 10-minute time point was obtained in this medium. When NaCl was added to increase the concentration of Na^+ by 50 mM, there was little or no effect on prolactin release (Fig. 1). We have shown previously that TRH also enhances the release of prolactin from GH_4C_1 cells within 10 minutes (18).

To determine whether the effect of cobalt on prolactin release by GH_4C_1 cells was due to its known action as a competitive calcium antagonist (12,13) two sorts of experiments were performed. First, the ability of calcium to reverse the inhibition due to cobalt was examined, and secondly, the effect of the calcium chelator EGTA was investigated in the absence and presence of excess calcium. When 2.0 mM $CaCl_2$ was added to either F 10(-) + 1h or F 10 (+) medium, there was no change in the spontaneous release of prolactin (Table 3). However, there was partial reversal by calcium of the antagonism of the high K^+ effect by cobalt in both media, and partial reversal by calcium of the antagonism of TRH-stimulated prolactin release by cobalt in F 10 (-) + 1h medium (Table 3). It is likely that reversal of the cobalt antagonism by calcium was incomplete because the concentration of calcium added was equimolar to that of cobalt. Nevertheless, partial reversal by calcium is consistent with the hypothesis that cobalt is acting as a competitive calcium antagonist in these experiments as it does in other systems (12,13). Additional evidence to support the importance of calcium in the en-

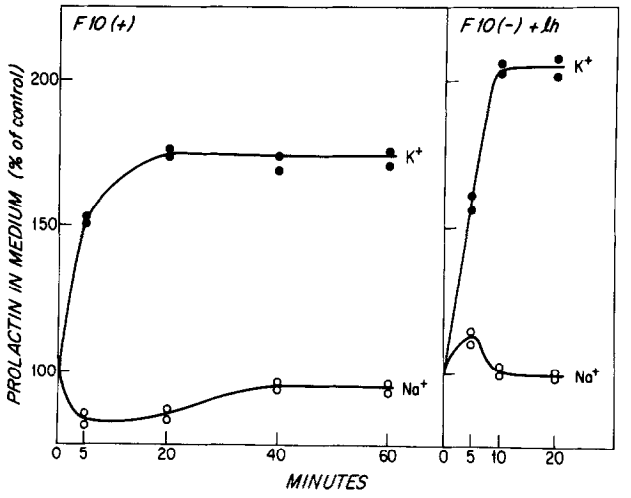

Fig. 1. Time-course of the effects of added (50 mM) KCl and NaCl on the release of prolactin from GH_4C_1 cells. Three groups of cultures were studied in both F 10(+) and F 10(-) + 1h medium. At zero time, the medium was changed on all dishes to the appropriate experimental medium to which was added nothing (control), 50 mM KCl (K^+) or 50 mM NaCl (Na^+). Medium from duplicate dishes was collected at the times indicated for measurement of prolactin concentration. The values presented are the changes from control cultures due to K^+ or Na^+ at each time interval sampled. Control medium prolactin concentration was 1,070 to 1,460 ng/ml in F 10(+) medium and 1,020 to 1,230 ng/ml in F 10(-) + 1h medium. Mean cell proteins in F 10(+) and F 10(-) + 1h media were 0.89 and 0.80 mg/dish, respectively; there were no significant differences between treatment groups in total cell protein/dish.

hancement of prolactin release stimulated by both high K^+ and TRH is given by experiments using EGTA. EGTA (1.0 mM) antagonized both the K^+- and TRH-stimulated release of prolactin, and this antagonism was reversed completely by addition of 2.0 mM calcium to the culture medium (Table 3).

DISCUSSION

We conclude from the results of the experiments presented in this communication that Ca^{+2} is an essential cation in mediating the actions of high external K^+ and TRH on prolactin release by GH_4C_1 cells. It is not likely that the effect of high K^+ was a nonspecific toxic action because it was blocked by relatively low concentrations of Co^{+2} (12,13) as well as by EGTA, and these antagonistic effects were reversed partially or completely by Ca^{+2}. A similar effect of EGTA on TRH-stimulated prolactin release from

Table 2

EFFECTS OF K^+, Co^{++} AND TRH ON THE RELEASE OF PROLACTIN

Treatment	Prolactin in medium (ng/ml)*	
	Spinner	F 10(+)
None	310 ± 5	400 ± 20
TRH (100 nM)	420 ± 30	780†
KCl (50 mM)	970 ± 0	900†
$CoCl_2$ (2.0 mM)	170 ± 10	260 ± 30
$CoCl_2$ (0.67 mM)	220 ± 10	320 ± 20
$CoCl_2$ (0.22 mM)	280 ± 20	380 ± 20
KCl (50) + $CoCl_2$ (2.0)	180 ± 5	400 ± 10
KCl (50) + $CoCl_2$ (0.67)	420 ± 15	760†
KCl (50) + $CoCl_2$ (0.22)	790†	970 ± 35
TRH (100) + $CoCl_2$ (2.0)	170 ± 10	380 ± 20
TRH (100) + $CoCl_2$ (0.67)	230 ± 20	550 ± 40
TRH (100) + $CoCl_2$ (0.22)	430 ± 10	830 ± 10

*Mean values from duplicate culture dishes ± range. The values given are those for prolactin accumulation in medium in 20 min. The mean protein concentrations per dish for cells in Spinner medium and F 10(+) were 0.47 and 0.37 mg, respectively.

†Single values, duplicate dish lost.

the GH_3 strain (15) of rat pituitary cells has also been observed (personal communication, P. S. Dannies). We presume that TRH and high K^+ act by enhancing entry of extracellular Ca^{+2} into cells via the nonNa^+, late Ca^{+2} channel which is blocked competitively in a variety of tissues by Co^{+2} as well as by Mg^{+2}, Mn^{+2}, La^{+3} and methoxyverapamil (1). On the other hand, our experiments do not rule out a redistribution of intracellular Ca^{+2} (2) as an additional action of TRH. Our experiments also do not permit a firm conclusion regarding the essentiality of external Ca^{+2} for basal prolactin release. Nevertheless, in all experiments performed, 1.0 to 2.0 mM $CoCl_2$ reduced basal release of prolactin (Tables 1-3), and EGTA had a similar effect in F 10 (-) + 1h medium but not in F 10(+).

The experiments reported here do not concern the uncertain role of cyclic AMP in the actions of TRH on prolactin and thyrotropin release (18,

Table 3

EFFECTS OF K^+, Co^{++}, TRH, EGTA AND Ca^{++} ON THE RELEASE OF PROLACTIN

Treatment	Prolactin in medium(ng/ml)*	
	F 10(-) + 1h	F 10(+)
None	190 ± 2	190 ± 2
TRH (100 nM)	310 ± 12	320 ± 5
KCl (50 mM)	455 ± 5	590 ± 30
$CoCl_2$ (2.0 mM)	115 ± 10	135 ± 5
EGTA (1.0 mM)	135 ± 10	205 ± 10
$CaCl_2$ (2.0 mM)	185 ± 15	220 ± 4
KCl + $CoCl_2$	165 ± 5	160 ± 5
KCl + $CoCl_2$ + $CaCl_2$	235 ± 10	195 ± 3
KCl + EGTA	205 ± 8	255 ± 25
KCl + EGTA + $CaCl_2$	485 ± 20	585 ± 30
TRH + $CoCl_2$	125 ± 12	170 ± 3
TRH + $CoCl_2$ + $CaCl_2$	175 ± 5	175 ± 9
TRH + EGTA	200 ± 20	220 ± 25
TRH + EGTA + $CaCl_2$	350 ± 45	420†

*Mean values from duplicate culture dishes ± range. The values given are for prolactin accumulation in medium in 20 min. The mean protein concentrations per dish for cells in F 10(-) + 1h and F 10(+) were 0.70 and 0.68 mg, respectively.

†Single value, duplicate dish lost.

20); however, they do relate directly to certain electrophysiologic studies with pituitary cells in culture. Kidokoro described a statistical effect of TRH on enhancing the frequency of calcium action potentials in the related GH_3 strain and concluded that "calcium ions traversing the membrane during action potentials may be related to the hormone secretion" by these cells (3). More recently, Taraskevich and Douglas reported action potentials in some primary mixed cultures of normal rat anterior pituitary cells which persisted in the presence of tetrodotoxin and in the absence of Na^+ but were inhibited by the calcium blockers D600 and La^{+3} (5). In about 10% of the cells on which it was tested TRH enhanced the frequency of action potentials or initiated them in previously electrically quiescent cells. These authors concluded

that "action potentials involving calcium influx participate in stimulus-secretion coupling" in these cells (5). Our results on prolactin release stimulated by TRH in GH_4C_1 cells and Vale's studies on thyrotropin release (7,8) are consistent with the proposals of Kidokoro (3) and Taraskevich and Douglas (5) as well as the more general Ca^{+2}-dependent stimulus-secretion coupling concept (1). On the other hand, the absolute frequency of positive electrophysiologic effects of TRH on pituitary cells in culture has been low and observed most readily with extracellular recording techniques, therefore additional experiments are needed before a functional relationship can be considered proven between TRH-stimulated, Ca^{+2}-dependent action potentials and TRH-mediated hormone release.

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