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Calcitonin Inhibits Prolactin Promoter Activity in Rat Pituitary GGH3 Cells

Evidence for Involvement of p42/44 Mitogen-Activated Protein Kinase in Calcitonin Action

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Previous findings from our laboratory have shown that pituitary calcitonin-like peptide (pit-CT) is synthesized and released by gonadotrophs and inhibits prolactin (PRL) release, synthesis, and lactotroph proliferation. To investigate further the regulation of PRL gene transcription by CT, we examined the effect of CT on rat PRL (rPRL) promoter activity in rat pituitary GGH3 cells. GGH3 cells were transiently transfected with rPRL promoter-luciferase and control plasmids. Thirty-six hours later, the cells were treated with CT or other agents and their effect on luciferase activity was examined. The effect of CT and/or thyrotropin-releasing hormone (TRH) on p42/44 mitogen-activated protein kinase (MAPK) activity was also investigated. CT inhibited basal rPRL promoter activity in a dose-dependent fashion, with an approximate IC_{50} of 3 n*M*. The maximal inhibition occurred 1 h after the CT addition, and the peptide was equipotent in inhibiting –600 and –2500 rPRL promoter constructs. CT also inhibited TRH-, Bay K 8644-, and ionomycin-induced rPRL promoter activity. CT mimicked the actions of MEK inhibitors U0126 and PD 980089. However, CT could not inhibit rPRL promoter activity in GGH3 cells expressing constitutively active ERK1 or ERK2. CT markedly attenuated phospho-MAPK immunoreactivity in untreated as well as TRH-treated GGH3 cells. These results suggest that CT inhibits rPRL promoter activity by antagonizing Ca^{2+} and ERK1/2-mediated signaling events. They also demonstrate that CT is a potent inhibitor of early events associated with PRL gene activation and may play an important role in regulation of lactotroph function.

Key Words: Prolactin promoter; regulation; calcitonin; mitogen-activated protein kinase.

Introduction

Calcitonins (CTs) are a group of polypeptide hormones containing 32 amino acid residues *(1–4)*. In addition to the thyroid gland, CTs are widely distributed in the central nervous system; the pituitary gland; and several other organs such as lung, uterus, and prostate *(5–11)*. Receptors for CT have also been detected in these organs *(12–15)*. CT exerts significant effects on the secretion and production of neurotransmitters and also alters growth and function of various target organs such as uterus, prostate, tuberoinfundibular dopaminergic neurons, as well as pituitary gland *(16–19)*. Thus, the diversity of sites of CT production as well as its actions suggests a variety of paracrine and autocrine roles for the peptide in addition to its originally described function of regulating of serum calcium.

CT-like pituitary peptide (Pit-CT) is synthesized and released by gonadotrophs of the anterior pituitary gland *(9,20, 21)*. Pit-CT has been suggested as a negative regulator of lactotroph function in rat anterior pituitary gland because of its selective, potent inhibition of prolactin (PRL) biosynthesis, secretion, and lactotroph cell proliferation *(22–24)*. Our earlier studies with rat pituitary GH3 cells showed that CT attenuates steady-state as well as thyrotropin-releasing hormone (TRH)–induced PRL mRNA levels in a dose-dependent manner by acting at the level of gene transcription *(23)*. Since pit-CT is secreted by gonadotrophs of the anterior pituitary gland in a highly regulated fashion *(25)*, CT can significantly influence lactotroph function in a paracrine manner. Consequently, pit-CT can affect various PRL-dependent events and may also alter the long-term development of the pituitary gland.

In the present study, we investigated the regulatory actions of CT on rat PRL (rPRL) promoter activity. We also examined the possibility that CT interferes with TRH-induced mitogen-activated protein kinase (MAPK) activation.

Results

CT Inhibits rPRL Promoter Activity, and Proximal Region of rPRL Promoter Is Sufficient for CT Action

Because CT has previously been shown to attenuate steady-state PRL mRNA abundance and PRL gene transcription in GH3 cells *(23)*, we tested its effect on rPRL promoter

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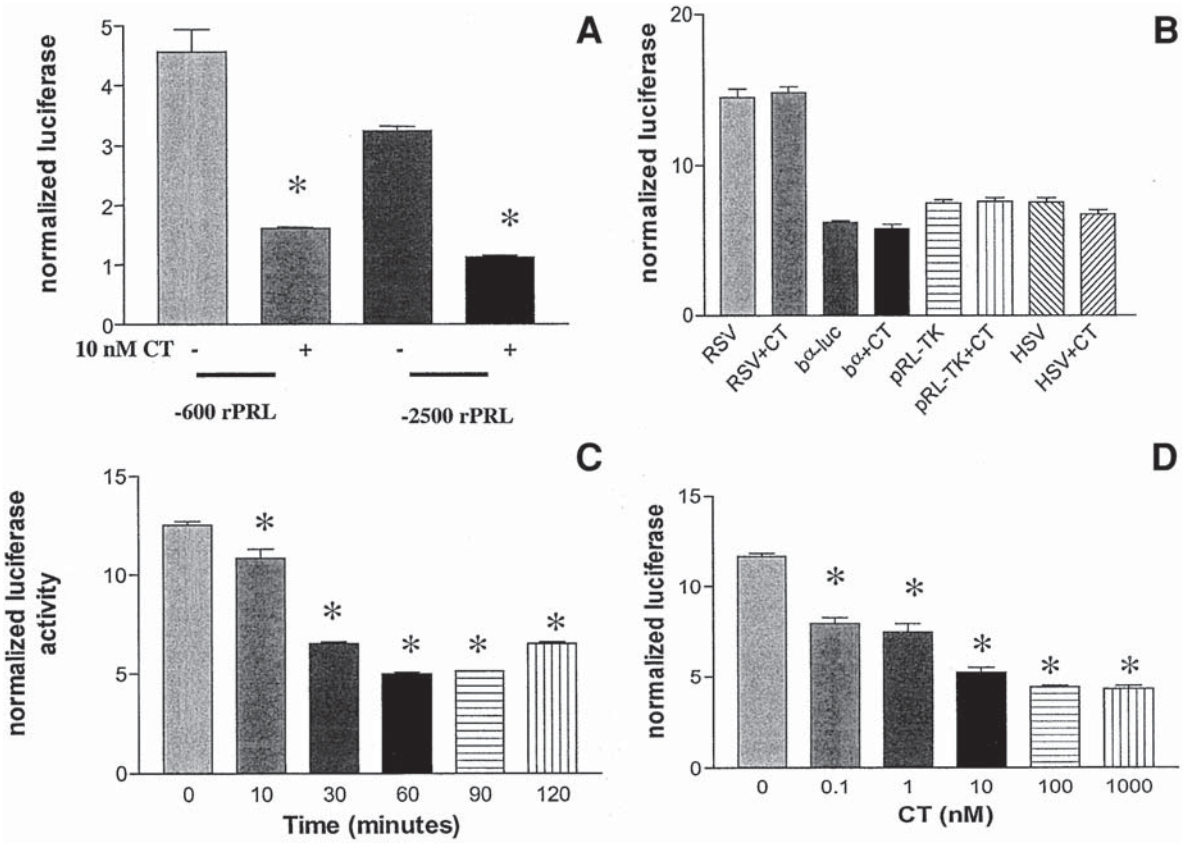

Fig. 1. CT inhibits rPRL promoter activity in time- and dose-dependent fashion. (**A**) Effect of CT on –600 and –2500 rPRL promoters. Exponentially growing GGH3 cells were transiently transfected with –600 (C1 and CT1) or –2500 rPRL luciferase and Renilla luciferase (pRL) constructs and cultured as described in Materials and Methods. After 36 h of culture, the cells were treated with or without 10 n*M* CT for 60 min. Normalized luciferase activity in cell lysates was calculated by dividing rPRL luciferase activity with Renilla luciferase activity. Data are expressed as mean ± SEM for a representative experiment done in quadruplicate. *$p < 0.05$ between the appropriate control and CT treated cells (one-way analysis of variance [ANOVA] and Newman-Keuls test). (**B**) Specificity of CT action on rPRL promoter activity. Exponentially growing GGH3 cells were transfected with other hormonal or viral promoters such as RSV, bovine α-subunit luciferase or HSV-tet-luciferase constructs. Indeed, Renilla luciferase was cotransfected in all treatment groups. The cells were then treated with 10 n*M* CT for 60 min as described in Materials and Methods. Normalized luciferase activity was calculated as described in (A), and the results are presented as mean ± SEM for $n = 4$. (**C**) Time course of CT action on rPRL promoter activity. GGH3 cells were transfected with –600 rPRL and pRL constructs as previously described and incubated with 10 n*M* CT for various time points up to 150 min. Normalized luciferase activity is presented as mean ± SEM for $n = 4$. *$p < 0.05$ between control (0 time point) and CT treated cells (one-way ANOVA and Newman-Keuls test). (**D**) Dose response. GGH3 cells were transfected as described above and treated with various concentrations of CT for 60 min. Data are presented as mean ± SEM of $n = 4$. *$p < 0.05$ between the control and CT treated cells (one-way ANOVA and Newman-Keuls test).

activity. We used two separate –2500 and –600 rPRL promoter-luciferase constructs because rPRL promoter contains distal (between –1561 and –1016 relative to the transcriptional start site) and proximal (between –326 to +73 relative to the transcription start site) enhancer regions *(26,27)*. The results presented in Fig. 1A show that 10 n*M* CT was equipotent in inhibiting –2500 as well as –600 rPRL promoter constructs. CT caused a 67% decrease in luciferase activity of both promoters. Therefore, all subsequent experiments used only proximal (–600) promoter construct.

Inhibitory Action of CT on rPRL Promoter Is Specific

Although CT affected rPRL promoter-luciferase activity, it did not alter either RSV 400, HSV-Tet-luc, bovine α-luteinizing hormone subunit-luc, or pRL-TK promoters (Fig. 1B). Our earlier studies on PRL secretion have shown that the action of CT was specific on lactotrophs and did not affect secretory activity of other anterior pituitary cell types *(21)*.

Inhibitory Action of CT on rPRL Promoter Is Rapid

The results presented in Fig. 1C show that 10 n*M* CT induced a rapid decrease in –600 rPRL promoter-luciferase activity. The initial decline was seen after 10 min, reaching nadir at 60 min. Longer incubation did not cause any greater inhibition. A maximal inhibition of 63% was achieved after 1 h of incubation.

CT Inhibits rPRL Promoter Activity in Dose-Dependent Manner

In the next group of experiments, the dose dependence of CT action on –600 rPRL promoter-luciferase activity was examined. The results in Fig. 1D show the effect of various

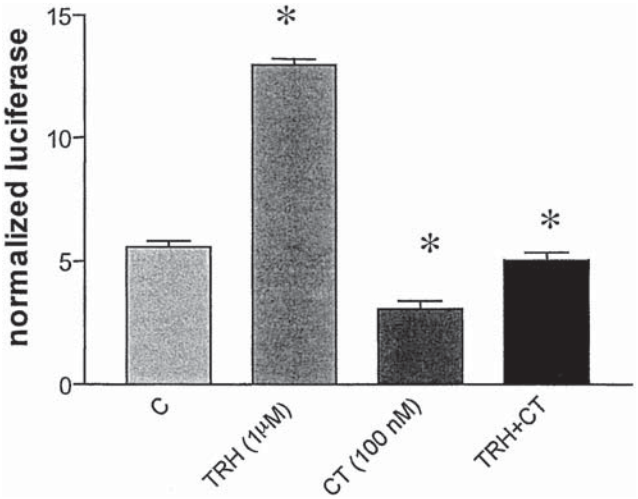

Fig. 2. Effect of CT on basal and TRH-induced rPRL promoter activity. GGH3 cells transfected with –600 rPRL and pRL constructs were treated with either 100 n*M* CT or 1 μ*M* TRH or a combination for 30 min. The results are presented as mean normalized luciferase ± SEM for $n = 4$. *$p < 0.05$ between the control and CT-, TRH-, or CT+TRH-treated cells (one-way ANOVA and Newman-Keuls test).

concentrations of CT on –600 rPRL promoter activity after 60 min of incubation. CT-induced decrease in the luciferase activity was dose dependent, and a maximal inhibition of 67% over the control was observed at a dose of 100 n*M* CT. The inhibitory action of CT was steep initially, and almost 25% inhibition of rPRL promoter activity was seen with 0.1 n*M* CT. A greater inhibition of 32% was obtained with 1 n*M*, and a further decline of 56% was observed with 10 n*M*. A maximal inhibition of 67% was observed with 100 n*M* CT, and 1000 n*M* CT did not produce greater inhibition. The calculated IC_{50} of CT was approx 3 n*M*. This IC_{50} is in the range of K_d for high-affinity CT receptors, which are in the 0.1–10 n*M* range *(28)*. All subsequent experiments used the IC_{50} concentration of CT (3 n*M*) unless otherwise stated.

Stimulation of rPRL Promoter Activity by TRH and Its Inhibition by CT

Transiently transfected GGH3 cells with –600 rPRL luciferase were treated with either 1 μ*M* TRH or 100 n*M* CT for 30 min. The results presented in Fig. 2 show that TRH caused an approx 2.5-fold increase in luciferase activity. This stimulation is less than that demonstrated by other investigators *(29)*, which may be owing to the shorter stimulation periods of our studies. CT at 100 n*M* significantly inhibited basal and TRH-stimulated rPRL promoter-luciferase activity. It is also possible that GGH3 cells may not have sufficient *trans*-acting factors to optimally activate endogenous as well as transfected PRL promoters in response to high concentrations of TRH. Additional studies will be necessary to examine this possibility further.

Inhibitory Action of CT on rPRL Promoter Activity: A Role for Ca^{2+}

Since cytoplasmic Ca^{2+}, protein kinase C (PKC), and Ca^{2+} channel activity play a major role in basal as well as TRH-induced PRL gene expression *(27–29)*, we examined the possibility that CT may inhibit rPRL promoter activity by affecting either of these paradigms. The results presented in Fig. 3A show that 10 n*M* Bay K 8644 stimulated rPRL promoter activity by 2.5-fold. CT (3 n*M*) significantly inhibited it by 35%. However, Bay K 8644–induced stimulation of rPRL promoter activity in the presence of CT was significantly lower. Similarly, ionomycin stimulated rPRL promoter-luciferase activity by more than twofold. CT (3 n*M*) inhibited it by 35% (Fig. 3B). Again, the ability of ionomycin to stimulate rPRL promoter luciferase activity was significantly attenuated by CT. Phorbol-12-myristate acetate (PMA) (100 n*M*) induced a similar increase in rPRL promoter activity in the presence or absence of CT (Fig. 3C). However, its effects on rPRL promoter activity were much smaller than those seen with Bay K 8644 or ionomycin.

Inhibition of Basal and TRH-Induced rPRL Promoter Activity by CT: A Role for MAPK Pathway

Since CT antagonized TRH actions on rPRL promoter activity, it is likely that CT may affect down stream signaling events activated by TRH receptor. Recent evidence suggests that the activation of MAPK (MAPK-ERK1/2) plays a critical role in TRH-induced increase in PRL promoter activity *(29)*. Therefore, we examined the possibility that CT may interfere with TRH-induced MAPK activation. In the initial experiments, we examined whether MEK inhibitors U0126 and PD 980089 mimic the action of CT on rPRL promoter activity, and whether their effects are additive with CT. The results presented in Fig. 4A show that the negative control for MEK inhibitor (U0124) did not affect either the basal rPRL promoter activity or the action of CT. However, MAPK inhibitor U0126 (100 n*M*) as well as CT (3 n*M*) inhibited rPRL promoter activity by 35%. When added together, these two compounds produced 60% inhibition, suggesting an additive effect at these concentrations. We also tested the effects of another MEK inhibitor, PD 980089, on rPRL promoter-luciferase activity. CT (3 n*M*) produced 34% inhibition in rPRL promoter activity. PD 980089 (100 n*M*) produced 49% inhibition. When added together, the combined inhibition was 69%. Even at a higher concentration of PD 980089 (1 μ*M*), CT produced further inhibition of rPRL promoter activity (74% inhibition with PD 980089 alone vs 82% with CT+PD 980089; Fig. 4B).

In a subsequent experiment, GGH3 cells were transfected with plasmids expressing constitutively active forms of ERK1 and ERK2 in addition to –600 rPRL-luc and pRL-luc. The cells were then treated with either CT or vehicle. The results presented in Fig. 4C show that 3 n*M* CT inhibited PRL promoter activity by 43%. However, CT could not inhibit PRL

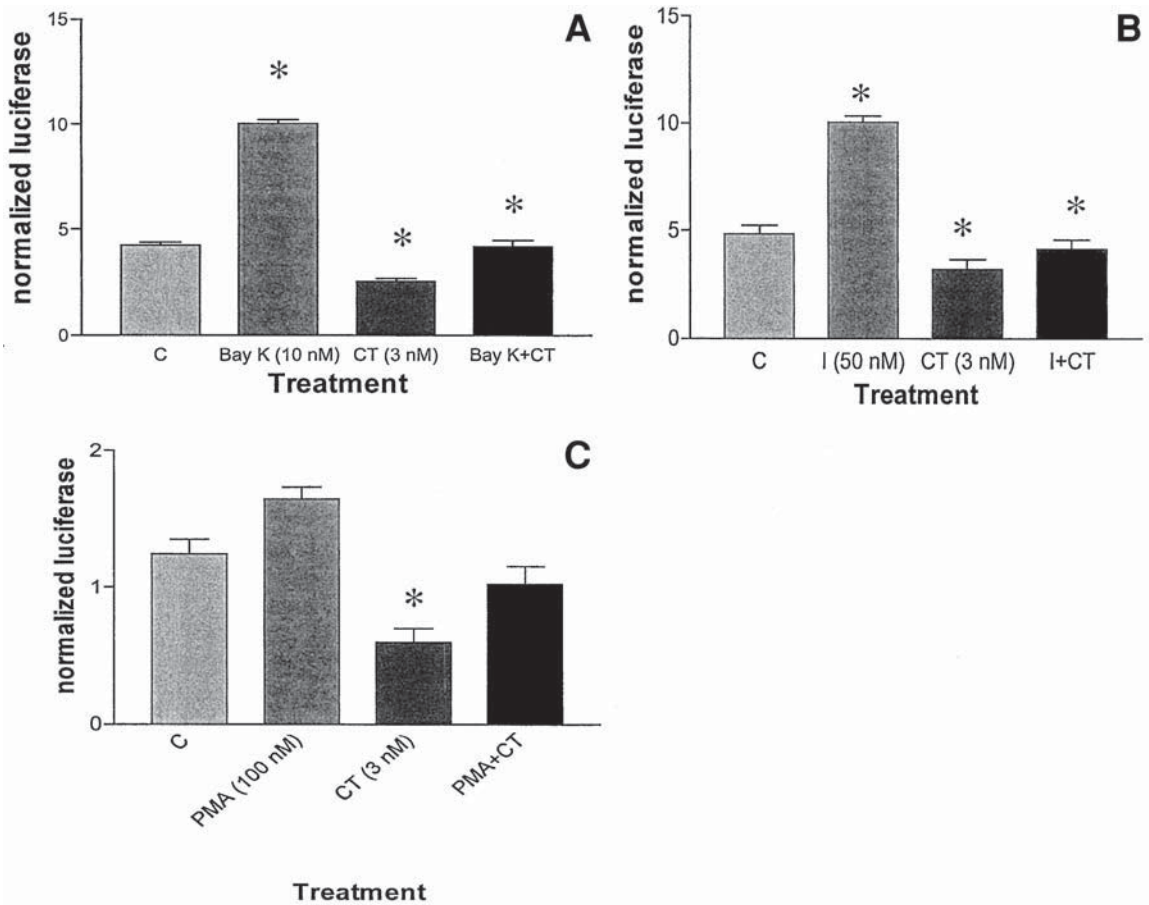

Fig. 3. Effect of cytoplasmic Ca^{2+} modulators on rPRL promoter activity: its modulation by CT. GGH3 cells were transfected with –600 rPRL and pRL promoter constructs and were treated with either (**A**) Bay K, (**B**) ionomycin (I), or (**C**) PMA in the presence or absence of 3 n*M* CT for 60 min. The results are presented as mean normalized luciferase activity ± SEM for $n = 4$. *$p < 0.05$ between the control and CT-treated cells (one-way ANOVA and Newman-Keuls test).

promoter activity in cells expressing constitutively active ERK1 or ERK2.

CT Attenuates Basal as well as TRH-Induced Increase in Phospho-MAPK Immunoreactivity

We examined the effect of CT on basal and TRH-induced ERK1/2 phosphorylation. The inhibitory action of CT on MAPK phosphorylation was relatively rapid, and the maximal inhibition was observed within 2.5 min of incubation (Fig. 5A). The CT-induced inhibition of MAPK gradually decreased with time. However, even after 60 min of CT incubation, phospho-MAPK immunoreactivity was still markedly lower than in untreated control cells. Moreover, CT did not alter total MAPK immunoreactivity at all time points investigated. The results presented in Fig. 5B show that CT markedly attenuated basal phospho-MAPK immunoreactivity of GGH3 cells in a dose-dependent fashion without affecting total MAPK immunoreactivity. As expected, 1 μ*M* TRH caused a dramatic increase in phospho-MAPK immunoreactivity when examined at 10 or 30 min (Fig. 6A). Similarly, CT attenuated (100 n*M*) phospho-MAPK immunoreactivity. When added together with TRH, CT almost abolished the TRH-induced increase in phospho-MAPK immunoreactivity at the 10- as well as 30-min time points. However, the inhibitory action of CT on TRH-induced MAPK phosphorylation was much weaker at the 60-min time point (Fig. 6B).

Discussion

CT has been reported as a gonadotroph-derived, potent, selective, and physiologically relevant inhibitor of PRL gene transcription and secretion in rat anterior pituitary gland *(19,21,22,30,31)*. The present studies extend these observations by demonstrating that CT inhibits PRL promoter activity rapidly and in a dose-dependent fashion. The results also suggest that the distal enhancer region of rPRL promoter is not essential for the action of CT on PRL promoter activity, and all functionally relevant CT-responsive site(s) may reside within the –600 to +73 region.

Subsequent experiments focused on delineating possible signaling pathways associated with the actions of CT on rPRL promoter activity. It has been shown that basal expression of PRL gene is dependent on cytoplasmic Ca^{2+} levels, volt-

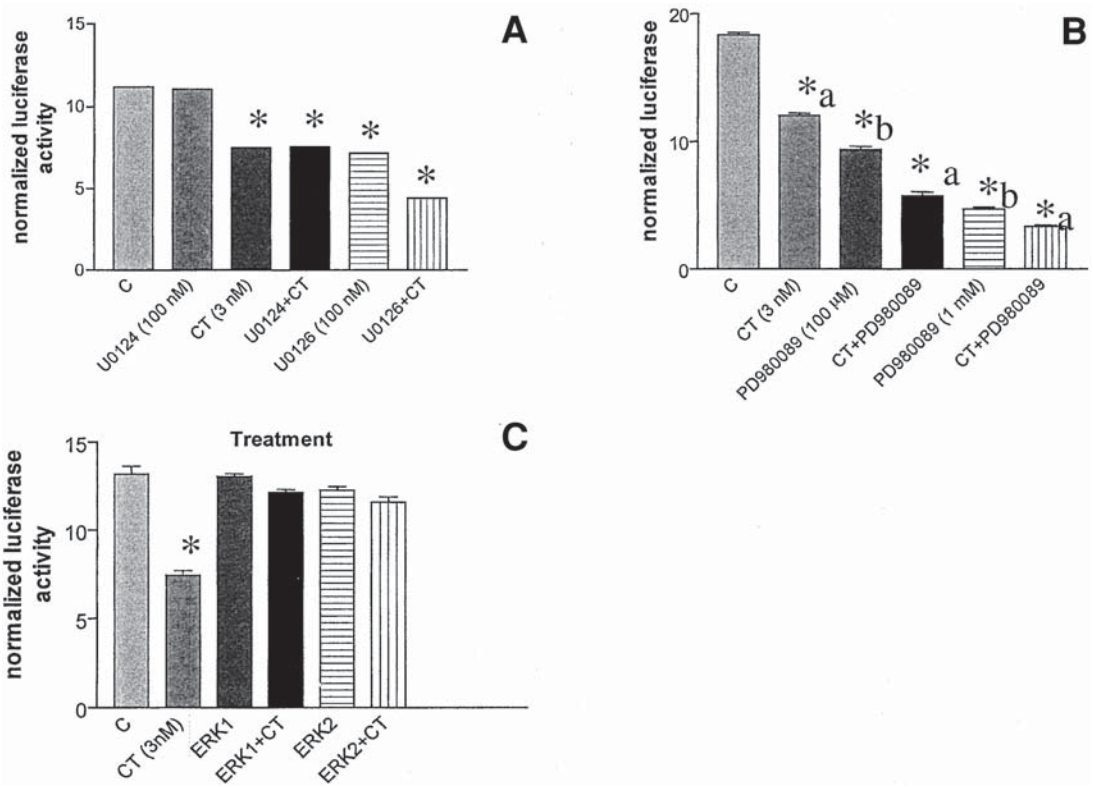

Fig. 4. Effect of MEK inhibition on rPRL promoter activity: its modulation by CT. GGH3 cells were transfected with –600 rPRL and pRL promoter constructs and were treated with either U0124 (control drug) or U0126 (MEK inhibitor) in the presence or absence of 3 n*M* CT for 60 min. The results are presented as mean normalized luciferase activity ± SEM for $n = 4$. *$p < 0.05$ between the control and treated cells (one-way ANOVA and Newman-Keuls test). (**B**) GGH3 cells were transfected with –600 rPRL and pRL promoter constructs and were treated with either 100 μ*M* or 1 m*M* PD980089 (MEK inhibitor) with or without 3 n*M* CT for 60 min. The results are presented as mean normalized luciferase activity ± SEM for $n = 4$. *$p < 0.001$ between the appropriate control and treated cells (one-way ANOVA and Newman-Keuls test). Comparisons were also made between various concentrations of PD 980089 as well as CT +/– PD980089. a,b: $p < 0.001$ between treatment groups (one-way ANOVA and Newman-Keuls test). (**C**) GGH3 cells were transfected with –600 rPRL, constitutively active ERK1 or ERK2, and pRL promoter constructs as described in Materials and Methods. The cells were then treated with or without 3 n*M* CT. The results are presented as mean normalized luciferase activity ± SEM for $n = 4$. *$p < 0.05$ between the appropriate control and treated cells (one-way ANOVA and Newman-Keuls test).

age-gated Ca^{2+} channel activities, as well as PKC *(32–34)*. Dihydropyridine derivatives, which selectively inhibit voltage-gated Ca^{2+} channels, inhibit PRL promoter activity in GH3 cells *(32)*. To investigate the role of these paradigms in CT-mediated inhibition of rPRL promoter activity, we examined the effects of CT on ionomycin-, Bay K 8644–, and PMA-induced stimulation of rPRL promoter activity. The results showed that CT did not attenuate PMA-induced stimulation of rPRL promoter activity. By contrast, CT significantly inhibited Bay K– and ionomycin-induced rPRL promoter activity. These results are consistent with the previous evidence that CT attenuates TRH-induced PRL release by interfering with TRH-induced increases in inositol phosphate generation *(33,35)*, and raise the possibility that it may interfere with ionomycin-induced redistribution of intracellular Ca^{2+} as well as the actions of Bay K 8644 on voltage-gated Ca^{2+} channels.

It has been shown that TRH induces rPRL promoter activity in rat pituitary GH3 cells, and this action may be mediated by TRH-induced increase in cytoplasmic Ca^{2+} and activation of PKC *(33,34,36–39)*. Since CT has been shown to attenuate TRH-induced increase in steady-state PRL mRNA levels, we tested the effect of CT on TRH-induced rPRL promoter activity *(19,22,23,40)*. The present results have also shown that CT caused a marked decline in TRH-induced rPRL promoter activity. Since the action of TRH on PRL promoter activity is mediated through the p42/44 MAPK signaling pathway *(29)*, we examined the role of MAPK in CT-induced inhibition of rPRL promoter activity. CT mimicked the actions of specific MEK inhibitors U0126 and PD 980089, and their actions were additive. However, in the presence of higher PD 980089 concentrations, CT was less effective in inhibiting rPRL promoter activity, suggesting that CT may affect MAPK activation. This was further examined in GGH3 cells transfected with constitutively active constructs of ERK1 and ERK2. CT could not inhibit PRL promoter activity in GGH3 cells when MAPK was activated. This reinforces the possibility that the action of CT on PRL

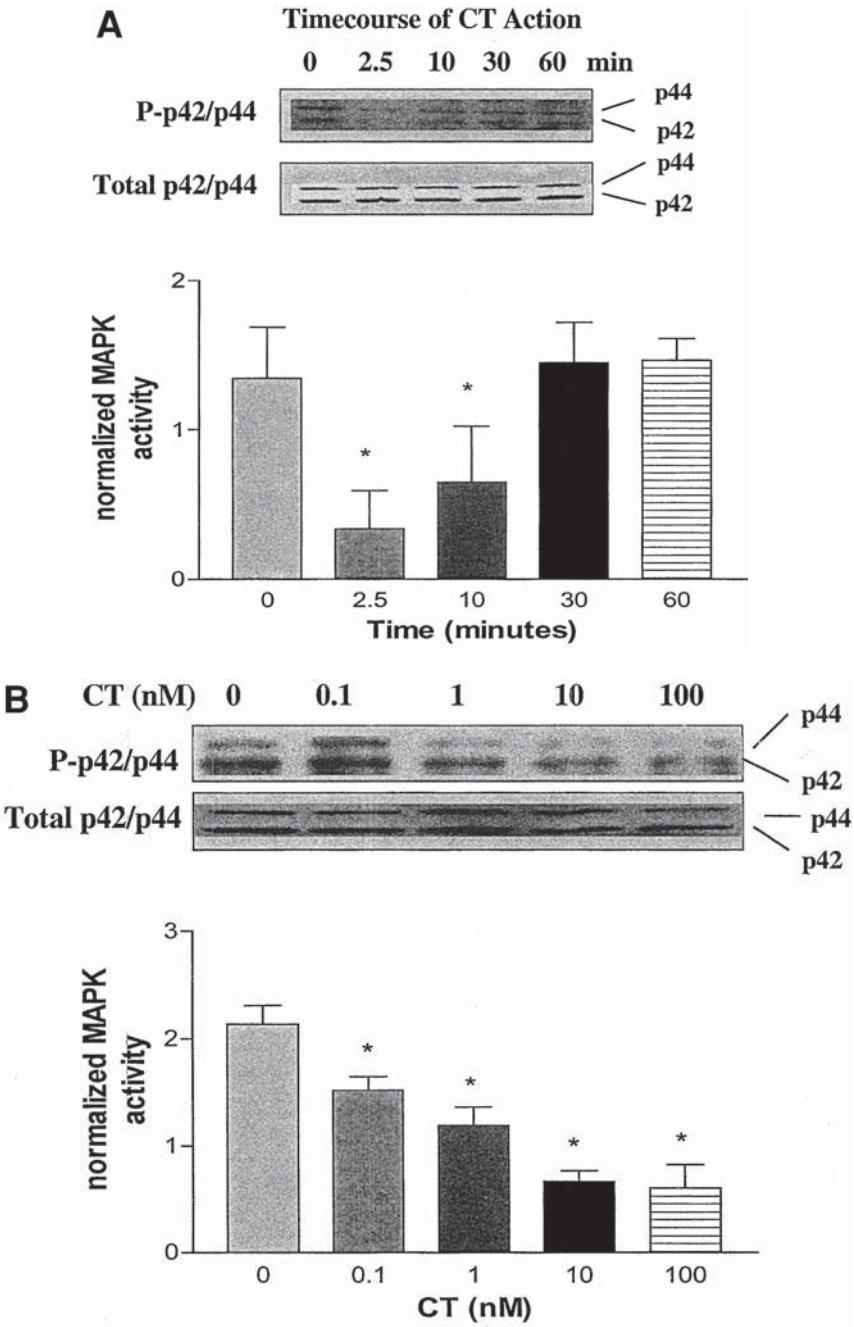

Fig. 5. Effect of CT on MAPK activity in GGH3 cells. (**A**) Time course. GGH3 cells were treated with 100 n*M* CT for various time points up to 60 min. The cells were then lysed and phosphorylated, and total p42/44 MAPK immunoreactivity was determined by Western blotting using specific antibodies (Cell Signaling, Beverly, MA). The data were then quantitated by determining the density of specific bands on autoradiograms in a Bio-Rad GS-700 imaging densitometer. A representative autoradiogram as well as mean ± SEM of densitometric values from four individual experiments is presented ($n = 4$). Significantly different from control: $*p < 0.05$ (one-way ANOVA and Newman-Keuls test). (**B**) Dose response. GGH3 cells were treated with various concentrations of CT for 2.5 min, and phospho- and total p42/44 MAPK immunoreactivity in the lysates was determined by Western blotting. The data were then quantitated by determining the density of specific bands on autoradiograms in a Bio-Rad GS-700 imaging densitometer. A representative autoradiogram as well as mean ± SEM of densitometric values from four individual experiments is presented ($n = 4$). Significantly different from control: $*p < 0.05$ (one-way ANOVA and Newman-Keuls test).

promoter activity may involve the ERK1/ERK2 pathway. Alternatively, there may be a crosstalk between the CT-activated inhibitory pathway and ERK1/ERK2. Therefore, we investigated the effect of CT on basal and TRH-induced phospho-MAPK immunoreactivity. As expected, CT markedly attenuated TRH-induced phospho-MAPK immunoreactivity, and this action was rapid, as evidenced by almost complete attenuation of TRH-induced MAPK activation within 10 min.

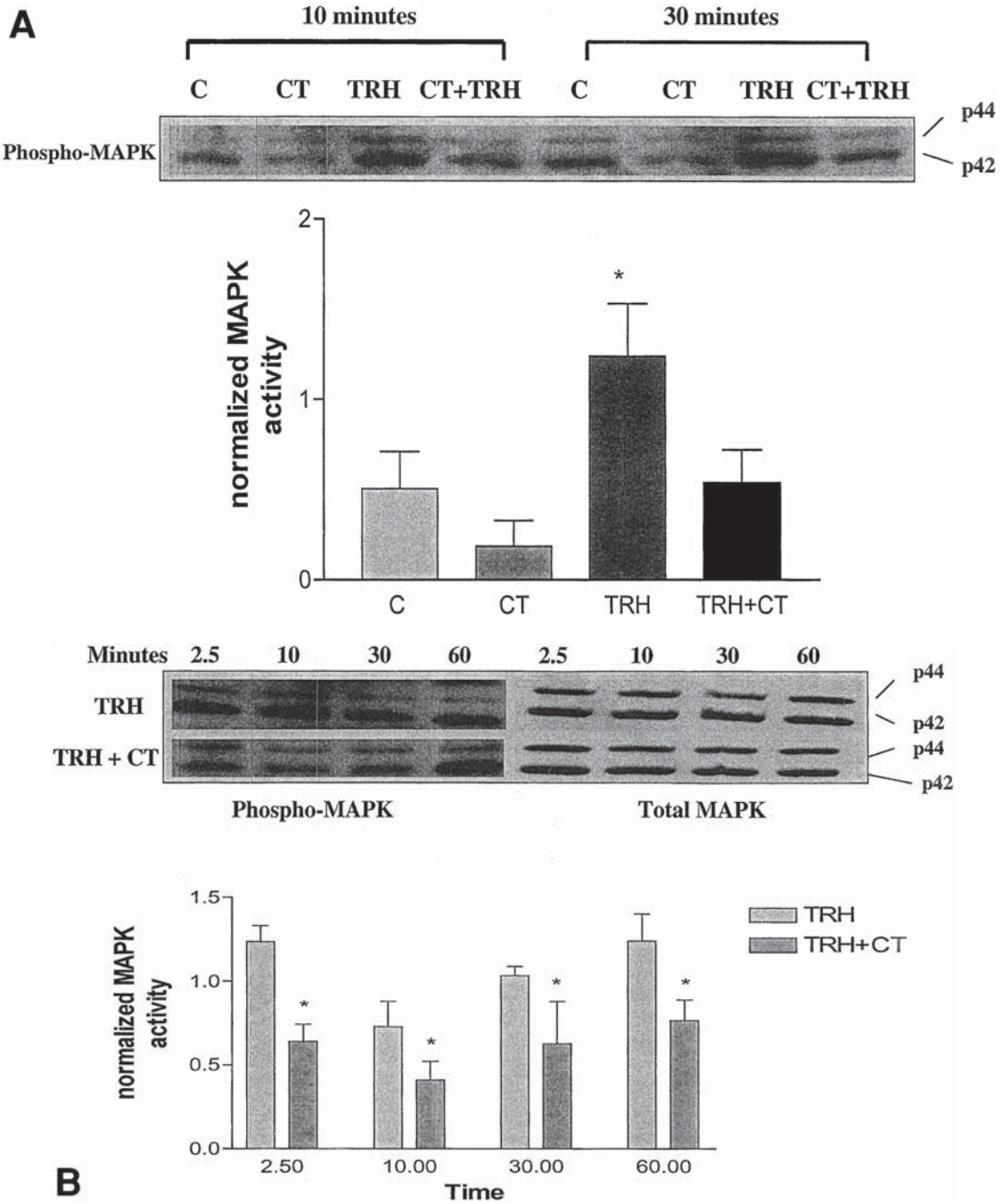

Fig. 6. Effect of CT on TRH-induced MAPK activity. (**A**) GGH3 cells were treated with either 100 n*M* CT, 1 μ*M* TRH, or a combination for either 10 or 30 min. The lysates were analyzed for phospho- and total p42/44 MAPK immunoreactivity by Western blotting. The data were quantitated by determining the density of specific bands on autoradiograms in a Bio-Rad GS-700 imaging densitometer. A representative autoradiogram as well as mean ± SEM of densitometric values from four individual experiments (incubation time of 10 min) is presented ($n = 4$). Significantly different from control: $*p < 0.05$ (one-way ANOVA and Newman-Keuls test). (**B**) GGH3 cells were treated with either 1 μ*M* TRH or 100 n*M* CT + 1 μ*M* TRH for various time points. The lysates were analyzed for phospho- and total p42/44 MAPK immunoreactivity by Western blotting. The data were then quantitated by determining the density of specific bands on autoradiograms in a Bio-Rad GS-700 imaging densitometer. A representative autoradiogram as well as mean ± SEM of densitometric values from four individual experiments is presented ($n = 4$). Significantly different from control: $*p < 0.05$ (one-way ANOVA and Newman-Keuls test).

In addition, the present results, for the first time, demonstrate that CT directly attenuates p42/44 MAPK activation in GGH3 cells. Taken together, the present results suggest that CT attenuates rPRL promoter activity, at least in part, by affecting MAPK activation. Additional studies will be necessary to delineate upstream as well as downstream events associated with the actions of CT on p42/44 MAPK activation.

In summary, our results demonstrate that CT rapidly and potently inhibits rPRL promoter activity in GGH3 cells in a dose-dependent fashion. This action of CT may involve intracellular Ca^{2+}, Ca^{2+} channel activity, as well as MAPK signaling pathway. Considering the evidence for the paracrine regulatory role for CT in the anterior pituitary gland, its potent action on PRL promoter activity suggests an important role for the peptide in regulation of lactotroph function.

Materials and Methods

Materials

Synthetic salmon (S) CT and TRH were purchased from Peninsula (Belmont, CA). Ionomycin, forskolin, PMA, Bay K 8644, and MAPK inhibitors U0124 and U0126 were purchased from Calbiochem. MEK inhibitor PD 900089 was obtained from New England Biolabs (Boston, MA).

Plasmids

–600 PRL-luc, –2500 PRL-luc, and mammalian expression vectors encoding constitutively active ERK1 and ERK2 sequences were kindly provided by Dr. R. Maurer of Oregon Health Sciences University. RSV-β-gal, RSV-luc, and HSV-TK were provided by Dr. Padmanabhan of the University of Kansas Medical Center. The pRL-TK Renilla luciferase control reporter vector was purchased from Promega (Madison, WI).

Cell Line

The GGH3 cell line, a variant of GH3 cell line stably expressing gonodotropin-releasing hormone (GnRH)-receptor, was kindly provided by Dr. Michael Conn of Oregon Health Sciences University. The cells were thawed and grown in Dulbecco's minimal essential medium supplemented with 10 m*M* HEPES, 15% horse serum, 5% fetal calf serum (FCS), 280 μg/mL of bacitracin, 100 U/mL of penicillin G–sodium, and 100 μg/mL of streptomycin sulfate.

Cell Culture, Electroporation, and Luciferase Assay

GGH3 cells (5,000,000 cells/mL) were transfected with plasmid DNA (30 μg/0.5 mL) by electroporation as described previously *(21)*. Some samples received either two plasmids (0.6 PRL-luc + RSV-β-galactosidase or 2.5 PRL-luc + RSV-β-galactosidase) or three plasmids (0.6 PRL-luc + ERK1/ERK2 + pRL-TK). RSV-β-galactosidase and/or pRL-TK were used as internal controls for the comparison of transfection efficiency. In brief, the cells received a single pulse of 240 V/0.4 mm, and 800 mF capacitance. The electroporated cells were seeded into six-well culture plates (200,000 cells/well in 2 mL of basal medium). The basal medium consisted of Dulbecco's minimal essential medium (pH 7.4) supplemented with 10 m*M* HEPES, 280 mg/mL of bacitracin, 100 U/mL of penicillin G–sodium, and 100 μg/mL of streptomycin sulfate. The cells were allowed to attach for 12 h, and the basal medium was replaced with the complete medium (basal medium supplemented with 15% horse serum and 5% FCS). After culture for an additional 24 h, the cells were washed with the incubation medium (basal medium containing 0.3% bovine serum albumin) and treated with CT or other agents for various incubation periods (30 or 60 min) as described in Results. At the end of incubation, the cells were harvested and lysed, and rPRL-luciferase, Renilla luciferase (Promega, Madison, WI) or β-galactosidase (Tropix, Bedford, MA) activities were determined according to the manufacturers' instructions. Luminiscence was measured in a Tecan multiwell plate luminometer. Each data point was in quadruplicate, and each experiment was repeated three times.

Statistical Calculations

A ratio of light units produced by rPRL luciferase to those produced by Renilla luciferase (or β-galactosidase) was determined for each sample and expressed as normalized luciferase activity. The results are presented as mean normalized luciferase activity ± SEM. The results were statistically evaluated by one-way ANOVA, and the significance was determined by Newman-Keuls test.

Immunoblotting and Determination of Phospho-ERK

GGH3 cells were grown to approx 80% confluency, transferred to serum-free basal medium, and then treated with hormones or drugs as described in Results. At the end of treatment, the cells were washed twice with ice-cold phosphate-buffered saline (0.15 *M* NaCl, 0.01 *M* $NaPO_4$, pH 7.4). The cells were lysed on ice in a 50 m*M* Tris buffer (pH 7.4, containing 1% NP-40, 0.25% Na-deoxycholate, 1 m*M* EDTA, and freshly supplemented with 1 m*M* phenylmethylsulfonyl fluoride, 1 μg/mL of leupeptin, 1 μg/mL of aprotinin, 1 μg/mL of pepstatin, 1 m*M* Na_3VO_4, and 1 m*M* NaF) for 10 min. The nuclear fraction and debris were separated by centrifugating at 2000*g* for 10 min at 4ºC, and the supernatant was used for Western blot analysis. Protein concentrations of the cell lysates were determined using Bio-Rad Reagent (Bio-Rad, Hercules, CA). The lysates were then boiled for 5 min in 2X Laemmli solution containing 20 m*M* dithiothreitol, and 50 μg of protein/lane was loaded on 12.5% sodium dodecyl sulfate polyacrylamide gel. The separated proteins were electrically transferred to a nitrocellulose membrane, and the blots were incubated with phospho-MAPK (ERK1/2) antiserum (1:1000; Cell Signaling) for 18 h at 4ºC. Following three washes, the membranes were incubated with anti–rabbit IgG-horseradish peroxidase (1:2000). Following three successive washes, the immune complexes were visualized on chemiluminescence radiography film using a Western blot ECL detection system (Radiochemical Center, Amersham). After radiography, blots were washed and reprobed with antiserum to MAPK (total, 1:1000; Cell Signaling) as just described. The same experiment was repeated two more times.

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