

## Protein kinase C regulates transcription of the human guanylate cyclase C gene

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Guanylate cyclase C is the receptor for the bacterial heat-stable enterotoxins and guanylin family of peptides, and mediates its action by elevating intracellular cGMP levels. Potentiation of ligand-stimulated activity of guanylate cyclase C in human colonic T84 cells is observed following activation of protein kinase C as a result of direct phosphorylation of guanylate cyclase C. Here, we show that prolonged exposure of cells to phorbol esters results in a decrease in guanylate cyclase C content in 4 $\beta$ -phorbol 12-myristate 13-acetate-treated cells, as a consequence of a decrease in guanylate cyclase C mRNA levels. The reduction in guanylate cyclase C mRNA was inhibited when cells were treated with 4 $\beta$ -phorbol 12-myristate 13-acetate (PMA) in the presence of staurosporine, indicating that a primary phosphorylation event by protein kinase C triggered the reduction in RNA levels. The reduction in guanylate cyclase C mRNA levels was not due to alterations in the half-life of guanylate cyclase C mRNA, but regulation occurred at the level of transcription of guanylate cyclase C mRNA. Expression in T84 cells of a

guanylate cyclase C promoter-luciferase reporter plasmid, containing 1973 bp of promoter sequence of the guanylate cyclase C gene, indicated that luciferase activity was reduced markedly on PMA treatment of cells, and the protein kinase C-responsive element was present in a 129-bp region of the promoter, containing a HNF4 binding element. Electrophoretic mobility shift assays using an oligonucleotide corresponding to the HNF4 binding site, indicated a decrease in binding of the factor to its cognate sequence in nuclear extracts prepared from PMA-treated cells. We therefore show for the first time that regulation of guanylate cyclase C activity can be controlled at the transcriptional level by cross-talk with signaling pathways that modulate protein kinase C activity. We also suggest a novel regulation of the HNF4 transcription factor by protein kinase C.

**Keywords:** guanylate cyclase C; HNF4; protein kinase C; transcription.

Guanylate cyclase C (GCC) is a receptor guanylate cyclase that binds the endogenous ligands, guanylin, uroguanylin and lymphoguanylin [1–5]. The heat-stable enterotoxin peptides (ST), secreted by pathogenic strains of *Escherichia coli* that cause watery diarrhea are super agonists of GCC, and interact with the receptor with higher affinity than the endogenous ligands [5]. GCC is expressed predominantly in the villus cells of the intestine, but expression has been detected in several other tissues such as the adrenal glands,

liver, stomach, kidney, olfactory mucosa, placenta, testis and airway epithelium [6–8]. Ligand binding to the extracellular domain of GCC [9,10] activates the cytoplasmic catalytic domain to cause a dramatic increase in intracellular cGMP levels. Elevated cGMP concentrations lead to phosphorylation and subsequent opening of the cystic fibrosis transmembrane conductance regulator (CFTR), via activation of cyclic GMP-dependent protein kinase [11], and in some cell lines through cross-activation of cAMP-dependent protein kinase [12]. Excessive Cl<sup>-</sup> secretion through CFTR results in the secretory diarrhea associated with the ST peptides [5]. A similar signaling cascade occurs in the human colonic carcinoma cell line, T84, which resembles mature intestinal cells in morphology and vectorial ion transport [13]. GCC cDNA has been cloned and the receptor characterized and purified from T84 cells [14,15].

A variety of mechanisms regulates GCC-mediated cGMP synthesis. Prolonged exposure of GCC to ST and uroguanylin in T84 cells leads to desensitization of GCC, exhibited by a reduction in cellular cGMP accumulation upon restimulation of ST [16]. The amount of cellular refraction to ST and uroguanylin peptides was attributed to both decreased cGMP synthesis, due to decreased catalytic activity of GCC [17], and increased cGMP degradation following the activation of the cGMP-specific,

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**Abbreviations:** CFTR, cystic fibrosis transmembrane conductance regulator; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assays; GCC, guanylyl cyclase C; HNF4, hepatocyte nuclear factor 4; PKC, protein kinase C; PMA, 4 $\beta$ -phorbol 12-myristate 13-acetate; ST, heat-stable enterotoxin; STh, heat-stable enterotoxin from human isolate; STY72F, ST with replacement of tyrosine at 19 amino-acid position in mature peptide with phenylalanine.

**Enzymes:** guanylate cyclase (EC 4.6.1.2); protein kinase (EC 2.7.1.37). (Received 13 September 2000, revised 30 January 2001, accepted 15 February 2001)

cGMP-binding phosphodiesterase, PDE5 [16,18]. Persistent exposure of GCC to ST or guanylin in membrane preparations also induces *in vitro* inactivation of GCC [19,20], which can be alleviated by the presence of ATP during ligand preincubation [21,22].

GCC-mediated signaling is also regulated by cross-talk with other signaling cascades. Pathways that activate protein kinase C (PKC) potentiate ST-stimulated guanylate cyclase activity *in vivo* [23–25]. PKC directly phosphorylates GCC on the Ser1029 residue and enhances ST-mediated GCC activation in terms of increased cGMP synthesis [26,27]. This ligand-dependent potentiation of GCC activity by PKC is also observed with the endogenous peptide, guanylin [20], suggesting that such cross-talk *in vivo* may have important physiological implications.

Regulation of GCC gene transcription may also serve as a means of modulating GCC activity in a cell. GCC mRNA is upregulated in the adult rat liver by injury and/or stress, leading to an acute phase response [28]. The function of hepatic GCC expression is unknown, but raises the possibility that in extra-intestinal tissues, modulation of mRNA levels could be a major mechanism in determining the efficacy of GCC-mediated signaling. The 5'-flanking region of the human GCC gene has been sequenced and the  $\approx 1.8$  kb sequence of the promoter reveals two TATA box-like sequences and binding sites present for several transcription factors such as Cdx2, hepatocyte nuclear factor 4 (HNF4), GATA-4, glucocorticoid receptor and NF-IL6 [29]. The role of these transcription factors in regulating GCC expression is not fully understood. However, in a recent report, it was shown that HNF4 regulates intestinal expression of GCC through an HNF4-binding element present between  $-50/-20$  bp of the GCC promoter [30].

In view of the possibility of regulation of GCC activity, both transcriptionally and post-transcriptionally, and the fairly ubiquitous distribution of PKC isoforms, the possible mechanism of regulation of GCC mRNA by activation of PKC was investigated. The studies reported here demonstrate that activation of PKC modulates not only the activity of GCC by direct phosphorylation, but also regulates the steady-state levels of GCC mRNA by downregulating GCC transcription. The interesting aspect of PKC-mediated regulation of GCC activity that emerges from this study is the dual, but contrasting, modulation of both GCC catalytic activity and its mRNA following PKC activation in T84 cells.

## MATERIALS AND METHODS

### Culture and maintenance of T84 cells

T84 cells were obtained from ATCC (CCL 247), and were maintained in Dulbecco's modified Eagle's medium (DMEM):F12 containing 5% new-born calf serum (Gibco BRL, Life Technologies, Inc., USA), 120 mg·L<sup>-1</sup> penicillin, and 270 mg·L<sup>-1</sup> streptomycin as described previously [31]. Cells were plated in 3-, 6- or 10-cm tissue culture dishes (Greiner, Germany) and were used at  $\approx 90\%$  confluence.

### Purification and radiolabeling of ST peptides

ST peptide was purified from the culture supernatant of a strain of *E. coli*, which overproduced the toxin, as

described previously [32,33]. Purified peptides were quantitated using amino acid analysis. An analog of STh, STY72F, was used as the radioligand for receptor binding analysis, and prepared as described previously [14].

### Preparation of membranes from T84 cells

Membranes were prepared from confluent cultures of T84 cells, following exposure of cells to the inactive phorbol ester 4 $\alpha$ -phorbol myristate acetate (4 $\alpha$  PMA; 100 nM; control) or 4 $\beta$ -phorbol 12-myristate 13-acetate (PMA; 100 nM) (Gibco BRL, Life Technologies Inc., USA) for the indicated periods. Confluent monolayers were harvested by scraping into 50 mM Hepes buffer, pH 7.5 containing 100 mM NaCl, 1 mM dithiothreitol, 5 mM EDTA, 2 mM PhCH<sub>2</sub>SO<sub>2</sub>F, 1  $\mu$ g·mL<sup>-1</sup> leupeptin, 1  $\mu$ g·mL<sup>-1</sup> aprotinin, 50 nM okadaic acid and 10 mM sodium orthovanadate as described previously [20]. Cells were sonicated in this buffer and subjected to centrifugation at 12 000 g for 1 h, at 4 °C and the crude membrane pellet thus obtained was taken for protein estimation by a modification of the Bradford method [34]. Membranes prepared this way were used for *in vitro* guanylate cyclase assays, receptor-binding assays and Western blot analysis.

### *In vitro* guanylate cyclase assays

*In vitro* guanylate cyclase assays were performed with membranes (50  $\mu$ g) prepared from control and PMA-treated T84 cells. Membranes were incubated in the presence or absence of STh (100 nM), in an assay buffer of 60 mM Tris/HCl buffer, pH 7.6, with 4 mM MgCl<sub>2</sub>, 1 mM GTP, 500  $\mu$ M 3-isobutyl methylxanthine (Sigma, St Louis, MO, USA) and a GTP-regenerating system consisting of 20  $\mu$ g creatine phosphokinase and 7.5 mM creatine phosphate (Sigma). Incubations were continued for 10 min at 37 °C and the reaction was terminated by addition of 400  $\mu$ L of 50 mM sodium acetate buffer, pH 4.0. Samples were boiled for 5 min and the supernatant taken for assay for cGMP by radioimmunoassay as described previously [31].

### Receptor binding assays

Binding of ST peptide to GCC was measured using <sup>125</sup>I-labeled STY72F as a radioligand as described previously [14]. Briefly, 50  $\mu$ g of membrane protein prepared from control or PMA-treated T84 cells was taken in 50 mM Hepes, pH 7.5, containing 4 mM MgCl<sub>2</sub>, 0.1% BSA, 10  $\mu$ g·mL<sup>-1</sup> leupeptin and 10  $\mu$ g·mL<sup>-1</sup> aprotinin, and incubated in the presence of varying concentrations of unlabeled STh peptide for 1 h at 37 °C along with  $\approx 100$  000 c.p.m. of <sup>125</sup>I-labeled STY72F in a total volume of 100  $\mu$ L. The radioactivity associated with the membrane fraction was monitored by filtration of the samples through Whatman GF/B glass-fiber filters, as described previously [14].

### Immunodetection of GCC in T84 cells by Western blot analysis

Membrane protein from T84 cells treated with 100 nM 4 $\alpha$ -PMA or 100 nM PMA for the indicated periods, was

fractionated on 7.5% SDS/polyacrylamide gels and the proteins were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech, UK). The nitrocellulose membrane was blocked with 5% blocking reagent for 1 h at 25 °C, after which the membrane was washed in 10 mM sodium phosphate buffer, pH 7.5, containing 0.9% NaCl (NaCl/P<sub>i</sub>) and 0.1% Tween 20 (NaCl/P<sub>i</sub>/Tween) and incubated for 2 h at room temperature with monoclonal antibody GCC:C8 raised to GCC at a concentration of 1 µg·mL<sup>-1</sup> in NaCl/P<sub>i</sub>/Tween containing 0.2% BSA [17]. Nitrocellulose membranes were subsequently washed extensively in NaCl/P<sub>i</sub>/Tween and further incubated with antimouse horseradish peroxidase conjugate (1 : 3000 diluted in NaCl/P<sub>i</sub>/Tween/BSA; Amersham Pharmacia Biotech) for 1 h. The presence of bound antibody was detected by enhanced chemiluminescence (ECL) reaction using the ECL Plus kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

### Northern blot analysis

T84 cells were treated with 4α-PMA, PMA (100 nM each) or carbachol (100 µM; Sigma) in the presence or absence of staurosporine (100 nM; Gibco-BRL, Life Technologies Inc.) for various times, in serum-free medium. In some cases, cells were also treated with 5 µg·mL<sup>-1</sup> actinomycin D (Roche, Germany) following PMA treatment. RNA was isolated using acid guanidium/isothiocyanate/phenol/chloroform according to the method of Chomczynski & Sacchi [35]. Total RNA (20 µg) from T84 cells was fractionated on 1% agarose-formaldehyde gels and transferred to Hybond-N membrane (Amersham Pharmacia Biotech). Prehybridization of the blots was performed in 0.5 M phosphate buffer, pH 7.0, 7% SDS, 1% BSA and 1 mM EDTA at 60 °C for 1 h [36]. To monitor GCC mRNA levels in T84 cells, a purified DNA fragment corresponding to the extracellular domain of human GCC cloned in pGEX-3X at the *SmaI*-*EcoRI* site (GCC:ED1) was used as a probe [9]. The DNA probes for hybridization were labeled with [α-<sup>32</sup>P]-dCTP (NEN, Dupont, USA) using the Megaprime DNA-labeling kit (Amersham Pharmacia Biotech). The radiolabeled probe was used at a concentration of 2 × 10<sup>6</sup> c.p.m.·mL<sup>-1</sup>, and hybridization performed at 60 °C for 16 h in the buffer used for prehybridization. Blots were washed twice with 2 × NaCl/Cit (300 mM NaCl, 30 mM sodium citrate, pH 7.0), and 0.1% SDS for 20 min at room temperature, twice with 0.2 × NaCl/Cit/0.1% SDS for 30 min at 60 °C, and exposed for autoradiography with intensifying screens, at -70 °C for 16 h. To normalize for equivalent RNA loading in the samples, blots were stripped of the hybridized GCC probe by washing extensively in 50 mM Tris/HCl buffer, pH 7.5 and then hybridized with a labeled DNA fragment corresponding to the human 18S rRNA. The mRNA levels were quantified by whole-band intensities using the Kodak Digital Science Imaging software and Kodak DC120 zoom digital camera.

### Nuclear run-on analysis

Confluent T84 monolayers were treated with 4α-PMA or PMA for 3 h. Nuclei were isolated by a modification of the method described by Greenberg & Ziff [37]. Approximately

5 × 10<sup>7</sup> cells were scraped into ice-cold buffer (10 mM Tris/Cl, pH 7.4, 3 mM MgCl<sub>2</sub> and 2 mM CaCl<sub>2</sub>) and pelleted by centrifugation at 500 g for 5 min at 4 °C.

The cells were lysed in the same lysis buffer containing 0.5% NP-40. Nuclear pellets were obtained by centrifugation at 500 g for 5 min at 4 °C, resuspended in 200 µL of glycerol storage buffer (50 mM Tris pH 8.3, 40% glycerol, 5 mM MgCl<sub>2</sub> and 0.1 mM EDTA) and stored at -70 °C until use.

To label nascent RNA transcripts, 200-µL aliquots of the nuclei (5 × 10<sup>7</sup>) were added to 200 µL of 2 × reaction buffer (10 mM Tris/Cl pH 8.0, 5 mM MgCl<sub>2</sub>, 0.3 M KCl, 1 mM each of ATP, CTP and GTP) and incubated with 250 µCi of [α-<sup>32</sup>P]-UTP (NEN Life Sciences) for 30 min at 30 °C. Nuclei were lysed in 0.5 M NaCl, 0.5 M Tris/Cl pH 7.4, 50 mM MgCl<sub>2</sub> and 2 mM CaCl<sub>2</sub>, containing 1% SDS, DNase I (5 U) and Proteinase K (1 mg·mL<sup>-1</sup>). RNA was isolated from the lysed nuclei using the hot phenol method of RNA extraction. Briefly, 2 vol. of phenol acidified with 1 M sodium acetate pH 4.4 was added to the lysed nuclei, heated at 55 °C for 5 min and subsequently chilled on ice. This was followed by the addition of 0.2 vol. of chloroform and the labeled RNA was precipitated from the aqueous phase by adjustment of the sample to 0.2 M NaCl, and the addition of 2 vol. of ice cold ethanol and 20 µg·mL<sup>-1</sup> yeast t-RNA. Samples were centrifuged at 12 000 g at 4 °C and the RNA pellet resuspended in 20 mM Hepes, pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> and treated with RNase-free DNase I (3 U) to remove residual genomic DNA. DNase I was inactivated by heating the sample at 65 °C for 10 min and unincorporated [α-<sup>32</sup>P]-UTP was removed by gel filtration through a Sephadex G-50 spun column.

Total denatured RNA was hybridized to cDNA fragments immobilized on nylon membrane. The entire coding sequence of human GCC obtained by restriction digestion of pcDNA3-hGCC with *XbaI* and *XhoI* [17], 500 ng of the complete cDNA for human 18S rRNA and 100 ng of human genomic DNA digested with *EcoRI*, were blotted directly onto the nylon membrane. Hybridization of labeled RNA to immobilized DNA was performed under conditions identical to Northern blot analysis as detailed earlier, at 65 °C for 42 h, after which the blot was washed twice with 2 × NaCl/Cit for 1 h at 65 °C. Unhybridized RNA was removed by treatment of the blot with RNase A (10 µg·mL<sup>-1</sup>) for 30 min at 37 °C. The blot was subsequently washed with 2 × NaCl/Cit for 1 h at 37 °C and specific transcripts were detected by autoradiography. The filters were exposed to Hyperfilm-MP (Amersham Pharmacia Biotech) for 72 h at -70 °C with intensifying screen and quantified as described above. Hybridization of radiolabeled RNA to genomic DNA was used to normalize for differences in incorporation of [α-<sup>32</sup>P]-UTP in different samples.

### Luciferase-reporter assays in T84 cells

The plasmid containing the human GCC promoter sequence fused to the firefly luciferase gene has been described previously [29,30]. Briefly, promoter fragments -1973/+124 and -128/+117 from the human GCC promoter were cloned into the pGL3-basic luciferase vector (Promega, Madison, WI, USA) to generate plasmids (-1973/+124)Luc

and (−128/+117)Luc, respectively. T84 cells were trypsinized 18–24 h prior to transfection and plated at ≈ 80% confluence in six-well dishes (Nunc). Dual luciferase reporter assays using GCC promoter–firefly luciferase constructs, and the pRL-TK vector (Promega), which contains *Renilla* luciferase under the control of the thymidine kinase promoter, were performed to normalize for transfection across individual wells. Independent experiments using the pRL-TK vector alone had shown that the activity of the thymidine kinase promoter was not affected by PMA treatment in T84 cells (data not shown). At the time of transfection, cells were washed in serum-free medium, and 1.5 mL of DMEM:F12 containing 5% fetal bovine serum was added to each well. Plasmid DNA was purified through a plasmid DNA purification kit (Quiagen, Germany). GCC promoter plasmids (2 μg) and pRL-TK plasmid (50 ng) were mixed with 3 μL of Fugene 6 transfection reagent (Roche Biomedicals) in 100 μL of antibiotic-free DMEM:F12 medium, as per the manufacturer's instructions. The mixture was kept for 15 min at room temperature and then added to individual wells containing T84 cells. Transfection was allowed to continue for 16 h in a CO<sub>2</sub> incubator, following which the cells were washed, and serum-free DMEM:F12 medium added with or without PMA (10<sup>−7</sup> M). Incubation was continued for 9 h, after which cells were washed in NaCl/P<sub>i</sub> and lysed in 60 μL of passive lysis buffer (Promega). Samples were centrifuged and 20 μL assayed with the Dual-Luciferase Reporter Assay System (Promega) as per the manufacturer's instructions. Measurements were made in a Turner Design Luminometer Model TD-20/20.

#### Nuclear extract preparation and electrophoretic mobility shift assay

T84 cells were either incubated with medium alone or PMA (100 nM) for 9 h at 37 °C. Nuclear extracts were prepared according to the procedure described by Swenson *et al.* [30]. Briefly, cells were rinsed with NaCl/P<sub>i</sub>, scraped and pelleted by centrifugation at 3000 g. Cells were resuspended in lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 0.2% NP-40, 1 mM dithiothreitol and 0.5 mM PhCH<sub>2</sub>SO<sub>2</sub>F). Nuclei were pelleted by centrifugation, rinsed with lysis buffer without NP-40, repelleted and then resuspended in extraction buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 25% glycerol, 1 mM dithiothreitol and 0.5 mM PhCH<sub>2</sub>SO<sub>2</sub>F). After 10 min on ice the extracted proteins in the supernatant were separated from insoluble nuclear debris by centrifugation at 14 000 g, and dialyzed against 20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol and 0.5 mM PhCH<sub>2</sub>SO<sub>2</sub>F. Samples were stored at −70 °C until use. Protein was estimated by a modification of the Bradford method [34].

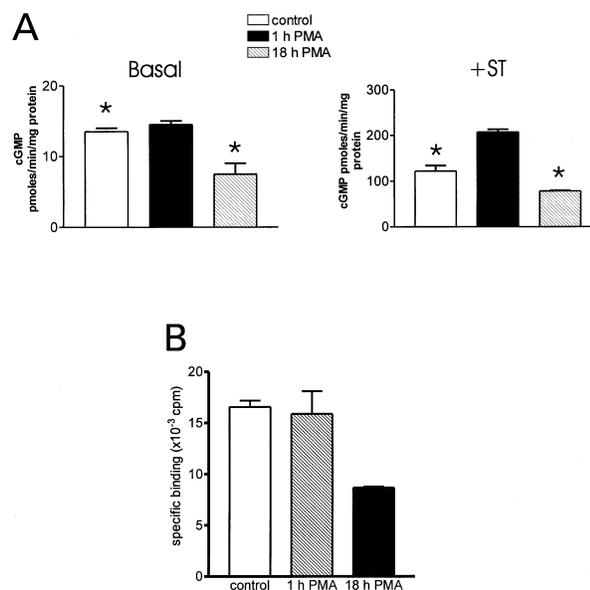
Electrophoretic mobility shift assays (EMSA) were performed according to the protocol described previously [30]. Oligonucleotides containing bases from −50 to −20 of the GCC promoter (5′-ACAAAGTGAACCTTGGTT-TATCTCCTGCCAT-3′) in both orientations were synthesized, annealed and labeled by T4 polynucleotide kinase using [γ-<sup>32</sup>P]-ATP (NEN Life Sciences). Unincorporated label was removed from the labeled double-stranded

oligonucleotide by gel filtration and taken for EMSA. Nuclear extracts (1 μg) were allowed to interact with 50 fmol (≈ 35 000 c.p.m.) of labeled oligonucleotide in binding buffer (20 mM Hepes, pH 7.9, 60 mM KCl, 12% glycerol, 1 mM dithiothreitol and 0.5 mM PhCH<sub>2</sub>SO<sub>2</sub>F) containing 250 ng of poly(dI-dC), in the presence or absence of 100-fold molar excess of unlabeled oligonucleotide. Interaction was continued for 10 min at room temperature and bound and unbound probe separated on a 5% polyacrylamide gel at 4 °C in 0.5× Tris/borate/EDTA. The gel was dried onto Whatman paper and subjected to autoradiography with an intensifying screen for 48 h at −70 °C.

## RESULTS

### Temporal regulation of GCC activity by PMA

A characteristic feature of many PKC-regulated phenomena is the fact that prolonged exposure to phorbol esters downregulates PKC activity, resulting in a loss of PKC-induced effects, initially seen after exposure to PKC-activating agents. Bearing this in mind, we wished to study the short- and long-term effects of PMA on GCC activity in human colonic T84 cells. T84 cells were therefore treated with 4α-PMA (control) or PMA for either 1 or 18 h. Upon 1 h PMA treatment, ST-stimulated synthesis of cGMP by



**Fig. 1.** Downregulation of GCC activity and ligand binding on PMA treatment of T84 cells. (A) Confluent monolayers of T84 cells were preincubated with 4α-PMA or PMA (100 nM) for the indicated times. Cells were washed, membranes prepared and used for *in vitro* guanylyl cyclase assays, either in the absence (basal) or presence of STh (100 nM). Values represent the mean ± SEM of duplicate determinations with each experiment performed three times. \**P* ≤ 0.05. (B) Binding assays were performed on membranes prepared from control and PMA-treated cells with <sup>125</sup>I-labeled STY72F as the radioligand. Nonspecific binding to T84 membranes was monitored in the presence of 10<sup>−7</sup> M unlabeled STh peptide and counts subtracted from the results shown. Values represent the mean ± SEM of duplicate determinations with each experiment performed three times.

GCC in intact cells was enhanced almost twofold (Fig. 1A). The potentiation of guanylate cyclase activity of GCC by 1 h PMA treatment that we observed was not because of increased ST binding to the receptor, as is evident from the results of receptor binding assays with  $^{125}\text{I}$ -labeled STY72F (Fig. 1B). Therefore, short-term treatment of T84 cells with PMA enhanced ligand-induced guanylate cyclase activity without a significant alteration in ligand receptor interaction [20]. In contrast, prolonged treatment (18 h) of T84 cells with PMA lead to a 50% reduction in ST-stimulated guanylate cyclase activity, and also a similar reduction in the basal activity of GCC (Fig. 1A). In addition, binding analysis with  $^{125}\text{I}$ -labeled STY72F (Fig. 1B) showed that 18 h treatment of T84 cells with PMA reduced the binding of radiolabeled STY72F to membranes prepared from these cells by 50%, indicating a reduction in GCC content.

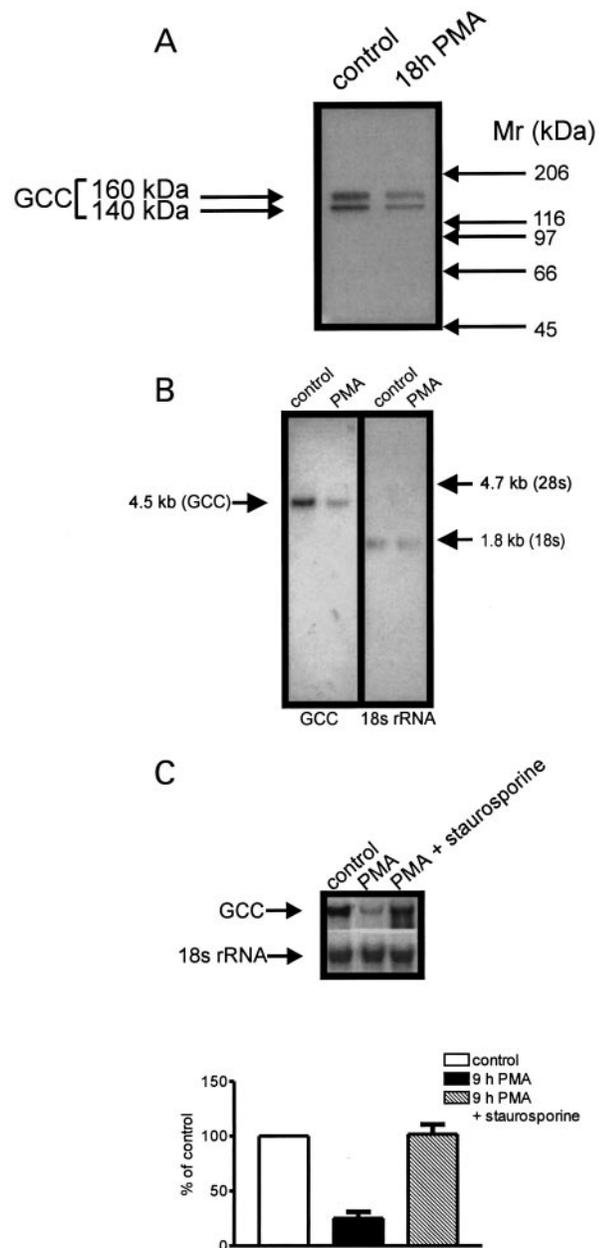
### PMA treatment leads to a reduction in GCC protein and mRNA in T84 cells

The decrease in receptor content in T84 cells was confirmed by Western blot analysis of membranes prepared from control and 18 h PMA-treated T84 cells using a GCC-specific monoclonal antibody GCC:C8. This antibody detects two bands of surface-associated, differentially glycosylated forms of GCC in T84 cells, with molecular masses of 140 and 160 kDa [17]. As shown in Fig. 2A, there was a decrease in the intensity of high-molecular mass bands corresponding to GCC in 18 h PMA-treated T84 cells. Densitometric analysis indicated a 50% reduction in the amount of both 140 kDa and 160 kDa fragments of GCC, which represent the functional forms of the receptor, following 18 h PMA treatment.

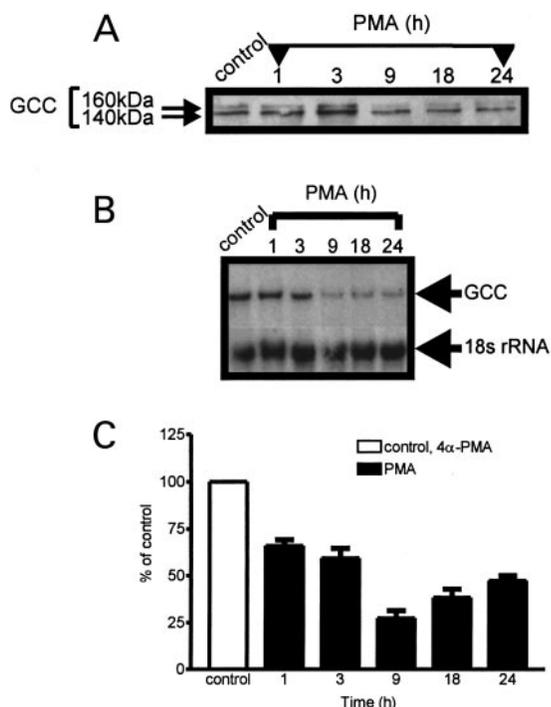
We hypothesized that this reduction in GCC content in T84 cells upon prolonged treatment with PMA could be due

to a reduction in GCC mRNA levels. To investigate this possibility, RNA was prepared from T84 cells treated with  $4\alpha$ -PMA and cells treated with 100 nM PMA for 18 h. Total RNA prepared from these cells was then subjected to Northern blot analysis using a GCC-specific probe corresponding to a fragment of the extracellular domain of human GCC cDNA. As shown in Fig. 2B, there was indeed a 60% decrease in GCC mRNA levels, indicating that a decrease in GCC receptor content seen on prolonged PMA treatment of cells was associated with downregulation of GCC mRNA.

Preincubation of T84 cells with 100 nM staurosporine prior to PMA treatment abolished PMA-mediated downregulation of GCC mRNA (Fig. 2C), indicating that the observed reduction in GCC mRNA was due to PMA-mediated activation of PKC and a subsequent phosphorylation event.



**Fig. 2.** PMA treatment of T84 cells leads to a reduction in GCC content and mRNA levels. (A) Changes in GCC content were measured by Western blot analysis using a monoclonal antibody to GCC. Membranes were prepared from T84 cells treated with either  $4\alpha$ -PMA or PMA and membrane protein (20  $\mu\text{g}$ ) subjected to SDS gel electrophoresis and blotted to nitrocellulose filters. Blots were then probed with GCC:C8 monoclonal antibody, and bound antibody detected using enhanced chemiluminescence. The differentially glycosylated forms of GCC ( $M_r$  140 000 and 160 000) are indicated. The data shown are representative of results obtained from three experiments. (B) Changes in steady-state mRNA levels were monitored by Northern blot analysis. Total RNA was prepared from T84 cell treated with  $4\alpha$ -PMA or PMA for 18 h, and subjected to agarose gel electrophoresis, and blotting onto a nylon membrane. Immobilized RNA was then probed with a labeled DNA fragment corresponding to the extracellular domain of human GCC. To normalize for RNA loading, the blot was re-probed with a DNA fragment corresponding to 18S rRNA. The data shown here are representative of three independent experiments. (C) T84 cells were treated with  $4\alpha$ -PMA (control) or PMA in the absence or presence of staurosporine (100 nM) for 9 h. Total RNA was prepared and subjected to Northern blot analysis as described previously. A representative blot of three independent experiments is shown here. The graph below the blot shows data normalized to 18S rRNA and GCC mRNA levels are expressed as a percentage of GCC mRNA in control T84 cells. Values represent the mean  $\pm$  SEM of three experiments.



**Fig. 3. Temporal changes in GCC protein and mRNA in PMA-treated T84 cells.** (A) Confluent monolayers of T84 cells were treated with 4 $\alpha$ -PMA for 18 h, or PMA (100 nM) for the indicated periods. Membrane protein was prepared from these cells and 50  $\mu$ g of protein subjected to Western blot analysis using the GCC:C8 monoclonal antibody. The data shown are representative of two independent experiments. (B) Total RNA was prepared from T84 cells treated with 4 $\alpha$ -PMA for 18 h (control) or PMA (100 nM) for various periods. RNA (20  $\mu$ g) was subjected to Northern blot analysis using a GCC-specific probe. RNA loading was normalized to the hybridization observed using a probe specific for the 18S rRNA. The data shown here are representative of three independent experiments. (C) Quantitation of Northern blot analysis data. GCC mRNA levels were normalized to 18S rRNA and expressed as a percentage of the hybridization observed in control cells. The mean  $\pm$  SEM for three independent experiments is shown.

#### Kinetics of decrease in GCC receptor and GCC mRNA on PMA treatment of T84 cells

To monitor temporal changes in GCC protein content and GCC mRNA, Western and Northern blot analysis was performed with membranes or total RNA prepared from T84 cells treated with PMA for varying times. Upon densitometric analyses of both the 140 and 160 kDa GCC immunoreactive bands, a slight increase in GCC protein was observed following 3 h of PMA treatment (Fig. 3A). This is in agreement with the increase in GCC content reported by other groups on PMA treatment for short periods [23,27]. Because there is no appreciable increase in GCC mRNA at this time (Fig. 3B), the increase in GCC content may represent more efficient translation of GCC mRNA, or a relocalization of preformed GCC within the cell. A decrease in receptor content, equivalent to a 60% reduction, was seen by the end of 9 h of PMA treatment and was still evident by the end of 24 h treatment.

In contrast, steady-state levels of GCC mRNA (Fig. 3B) were decreased after only 1 h treatment of T84 cells with PMA, with a maximum reduction (> 70%) in GCC mRNA levels detected after 9 h of treatment with PMA. Longer treatment with PMA (18–24 h) resulted in a recovery of GCC mRNA to 50% of that seen in control cells. We have shown earlier that 1 h PMA treatment of T84 cells led to potentiation of ST-stimulated GCC activity (Fig. 1A), but the data shown in Fig. 3A show no significant change in antibody-reactive GCC protein content at this time. Therefore, PMA treatment simultaneously upregulates GCC activity and downregulates GCC mRNA. Reduction in GCC receptor content is observed only after 9 h of PMA treatment, whereas a decrease in mRNA levels was observed earlier, indicating a relatively long half-life of GCC protein in T84 cells.

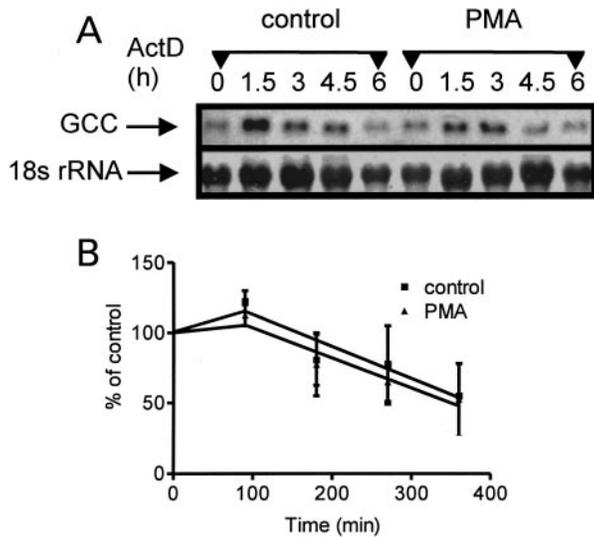
#### PKC activation does not decrease GCC mRNA stability but reduces GCC transcription in T84 cells

A decrease in GCC mRNA may result from either an increase in the rate of degradation of GCC mRNA or a decrease in the rate of transcription of the GCC gene. To determine the effect of PMA treatment on the degradation of GCC mRNA, T84 cells were first treated with 4 $\alpha$ -PMA or PMA for 2 h because our data indicated a significant decrease in mRNA levels by 3 h of PMA treatment (Fig. 3). Subsequently, actinomycin D was added to inhibit further transcription, and the levels of GCC mRNA at various times after addition of actinomycin D were measured by Northern blot analysis (Fig. 4). An initial increase in the stability of GCC mRNA following 90 min of actinomycin D treatment was seen in both control and PMA-treated cells. However, the rate of decrease of GCC mRNA content in both control and PMA-treated cells following this was similar. Analysis of the results indicated that the half-life of GCC mRNA was 6 h in both control and PMA-treated T84 cells. Thus, PKC activation did not appreciably alter the stability of GCC mRNA.

Next, we examined the possibility that the activation of PKC in T84 cells could directly reduce GCC transcription. Run-on analysis was performed with nuclei prepared from T84 cells treated with either 100 nM 4 $\alpha$ -PMA or 100 nM PMA for 3 h, again based on the assumption that significant changes in transcription should have been seen by 3 h, for levels of mRNA to show a reduction by 9 h (Fig. 3). Changes in transcription of the 18S rRNA gene were also monitored as an internal control. The results of the run-on analysis showed  $\approx$  25% reduction in transcription in the nuclei prepared from PMA-treated T84 cells, as monitored by hybridization of labeled transcripts to genomic DNA (Fig. 5). However, a 70% reduction in GCC transcription was observed on PMA treatment and, in contrast, a 10% increase in transcription of the 18S rRNA was observed. Therefore, we can conclude that the decreased steady-state levels of GCC mRNA observed following PKC activation were a result of reduced transcription of GCC mRNA.

#### Functional analysis of regulation of human GCC promoter by PKC

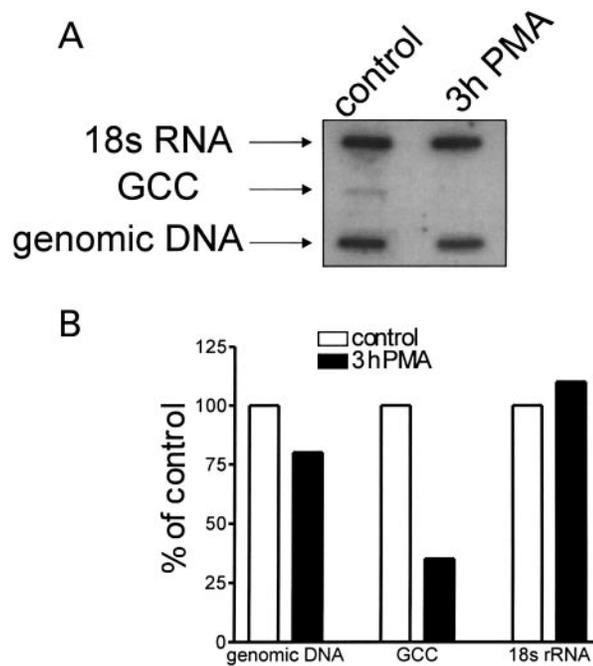
To identify possible promoter elements in the GCC gene that were regulated by PKC, plasmids containing sequences



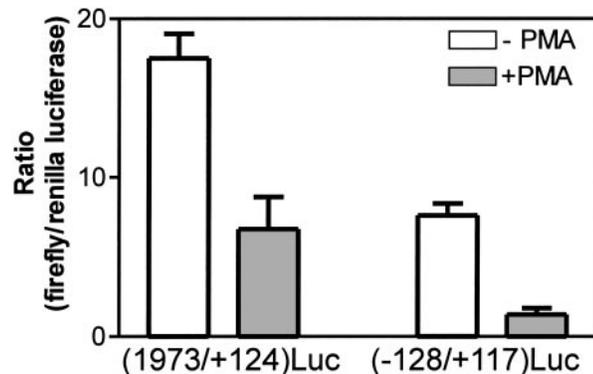
**Fig. 4.** Activation of PKC in T84 cells does not lead to a reduction in the half-life of GCC mRNA. (A) T84 cells were incubated with  $4\alpha$ -PMA or PMA for 2 h, followed by the addition of  $5 \mu\text{g}\cdot\text{mL}^{-1}$  of actinomycin D. Incubation was continued for the indicated times after actinomycin D addition, and total RNA was prepared and subjected to Northern blot analysis with GCC and 18S rRNA specific probes as described earlier. The blot shown is representative of three independent experiments. (B) The intensities of bands, monitored by phosphoimaging analyses, corresponding to GCC mRNA were normalized with respect to RNA loading and the data from three experiments analyzed. GCC mRNA levels had decreased to 30% of control T84 cells following 2 h PMA treatment at the time of actinomycin D addition (0 h) and the amount of GCC mRNA detected at 0 h in both control and 2 h PMA-treated cells was set at 100%. The data shown are the mean  $\pm$  SEM of three experiments.

from the 5'-region of the GCC gene (-1973/+124), as well as a sequence containing the proximal 129 bp (-128/+117) of the promoter [30] fused to the luciferase reporter gene, were transiently transfected in T84 cells and luciferase activity compared in control and PMA-treated T84 cells. The larger promoter sequence contains binding sites for several transcription factors, some of which are tissue specific. The proximal 129 bp of the GCC promoter appear to be the minimal requirement for detectable GCC transcription [30].

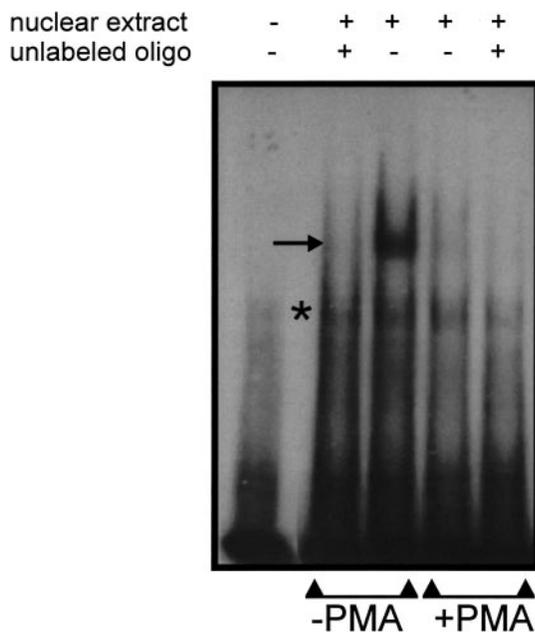
The pGL3-basic vector showed no detectable activity when transfected in T84 cells (data not shown). Both (-128/+117)Luc and (-1973/+124)Luc reporter plasmids were active when transfected in T84 cells but higher luciferase reporter activity was detected with the full-length GCC promoter, as reported earlier (Fig. 6) [29]. PMA treatment of T84 cells transfected with the (-1973/+124)Luc reporter construct led to a 60% decrease in luciferase activity, confirming that regulatory elements involved in PKC-mediated regulation of GCC transcription reside within this 1.8 kb of promoter sequence (Fig. 6). Interestingly, T84 cells transfected with the minimal 129 bp promoter construct (-128/+117)Luc, showed an even greater reduction (81%) in luciferase activity upon PMA treatment (Fig. 6). These results clearly indicate that GCC transcription is regulated by PKC through elements present in the 5' upstream promoter, suggesting that transcriptional



**Fig. 5.** Regulation of GCC gene transcription by PKC activation. (A) T84 cells were exposed to  $4\alpha$ -PMA or PMA for 3 h and nuclei prepared and subjected to run-on transcription assays as described in the text. Following autoradiography, the results were quantified by densitometric analyses of the bands on the X-ray film. A representative autoradiogram is shown of experiments performed three times and represents the hybridization observed with *in vitro* transcribed RNA to genomic DNA (100 ng), cDNA corresponding to entire GCC (3  $\mu\text{g}$ ) and the 18S rRNA (500 ng) immobilized on nylon filters. The blot shown is representative of three experiments. (B) Radioactivity associated with each band was quantitated and the data analyzed by considering the hybridization observed in control samples as 100%.



**Fig. 6.** PKC activation alters GCC promoter activity in T84 cells. T84 cells were transfected with plasmids (-1973/+124)Luc or (-128/+117)Luc encompassing GCC promoter elements, along with the pRL-TK vector to normalize for transfection efficiency across individual wells. Sixteen hours following transfection, cells were treated with  $4\alpha$ -PMA or PMA for 9 h, following which cell extracts were prepared and assayed simultaneously for firefly luciferase and *Renilla* luciferase (control vector) activities. The data shown are the ratio of the firefly luciferase activity to that of *Renilla* luciferase activity, and represent the mean  $\pm$  SEM of ratios obtained in three individual wells, with the experiment performed twice.



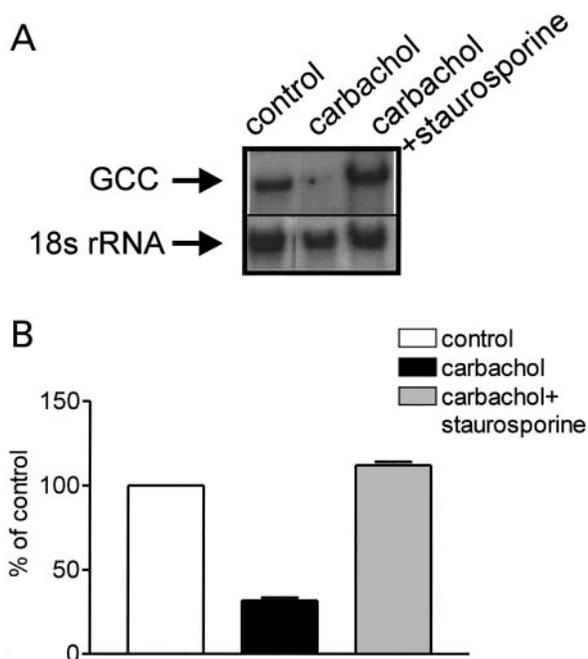
**Fig. 7. PKC activation inhibits the DNA binding activity of HNF4.** T84 monolayers were treated with  $4\alpha$ -PMA or PMA (100 nM) for 9 h. Nuclear extracts were prepared and EMSA was performed with 1  $\mu$ g of the nuclear extract and 35 000 c.p.m. (50 fmol) of a  $^{32}$ P-labeled oligonucleotide corresponding to the sequence -50/-20 in GCC promoter, either in the presence or the absence of unlabeled oligonucleotide. The arrow indicates the specific protein-DNA complex. The more rapidly migrating nonspecific complex is indicated by an asterisk. The data shown here are representative of three independent experiments with three independent nuclear extract preparations.

regulation of GCC could be a means of controlling GCC activity in differing cellular environments.

#### Role of HNF4 in PKC-mediated downregulation of GCC transcription

The extent of GCC transcription could be mediated by regulation of transcription factor binding to the GCC promoter. In view of the absolute requirement for functional HNF4 binding to activate GCC transcription [30], the hypothesis that PKC activation downregulates GCC transcription by altering the DNA-binding ability of HNF4 to its target site, was tested.

EMSA was performed with the oligonucleotide probe from the GCC promoter (-50/-20) containing the HNF4-binding site and nuclear extracts prepared from control and PMA-treated T84 cells. This probe formed two complexes with the nuclear extract prepared from control T84 cells (Fig. 7, lane 3). Specific complex formation could be blocked by addition of 20-fold molar excess of unlabeled -50/-20 GCC (Fig. 7, lane 2). Previous experiments had shown that this complex was due to HNF4 binding, using antibodies to HNF4 [30]. Formation of the second, more rapidly moving, complex could not be blocked by unlabeled -50/-20 GCC oligonucleotide. PMA treatment of T84 cells led to an almost complete disappearance of the complex of HNF4 transcription factor with its corresponding target site in the oligonucleotide (Fig. 7, lane 4). No change in the intensity of the nonspecific complex was



**Fig. 8. Carbachol treatment of T84 cells downregulates GCC mRNA.** (A) T84 cells were treated with carbachol (100  $\mu$ M) or medium alone for 9 h, either in the presence or absence of staurosporine (100 nM). Total RNA prepared from cells was subjected to Northern blot analysis using a GCC-specific probe and a 18S rRNA probe to normalize for RNA loading. Data are shown in a representative blot of experiments performed three times. (B) Band intensities were quantified by densitometric scanning, normalized with respect to 18S rRNA, and the intensity observed in the control was sent to 100%. Values represent the mean  $\pm$  SEM for duplicate determinations with experiments performed three times.

noted upon PMA treatment. Therefore, these observations clearly strengthen our hypothesis that the activation of PKC could lead to downregulation in GCC transcription by changes in the ability of HNF4 to bind its target site in the GCC promoter.

#### Carbachol treatment of T84 cells results in downregulation of GCC mRNA

The potentiation of ligand-stimulated activity of GCC by PMA is mimicked by carbachol, a cholinergic agonist of the  $M_3$  muscarinic receptor in T84 cells, which leads to phospholipase C activation and a subsequent increase in PKC activity [24]. We investigated whether carbachol treatment of cells could also mimic the downregulation of GCC mRNA via the activation of PKC. For this purpose, Northern blot analysis was performed with total RNA prepared from control T84 cells or cells treated with carbachol in the presence or absence of staurosporine. Treatment of T84 cells with carbachol for 9 h downregulated GCC mRNA (Fig. 8). In addition, carbachol-mediated GCC mRNA downregulation could be inhibited by preincubation of cells with staurosporine, indicating the involvement of PKC. These results therefore suggest that downregulation of GCC mRNA could occur *in vivo*, via signaling pathways that modulate PKC activity.

## DISCUSSION

In this study, we provide evidence that PKC can regulate GCC activity in T84 cells, not only by the earlier reported mechanism of direct phosphorylation [26,27], but also at the level of transcription of GCC mRNA. Earlier reports have provided evidence that GCC mRNA and protein are upregulated in the regenerating rat liver [28,38]. Here, we show that PKC activation in intestinal cells results in the downregulation of GCC mRNA and protein. Our studies using the T84 cell line as a model system provide opportunities to identify novel signaling cascades in the intestinal cell, involving receptors that regulate PKC activity and GCC.

In T84 cells, it has been reported that the major isoforms of PKC present in cells are the  $\alpha$ ,  $\delta$ ,  $\epsilon$  and  $\theta$  [27,39]. The  $\alpha$  isoform is downregulated, whereas the  $\delta$  isoform is upregulated and translocated to the membrane on prolonged exposure of cells to phorbol esters [39]. While the isoform responsible for GCC phosphorylation in T84 cells is not known, *in vitro* phosphorylation of GCC in immunoprecipitates has been demonstrated using as an enzyme source a fraction largely constituting the  $\alpha$  isoform [27]. It is interesting to note that ST is reported to increase PKC activity transiently in rat intestinal villus cells [40] as is adherence of enteropathogenic strains of *E. coli* [41], and these mechanisms may possibly provide a positive feedback loop allowing the potentiation of guanylin/uroguanylin action in the intestine.

Northern blot analysis demonstrates a maximal 70% reduction in GCC mRNA levels, preceding the 60% reduction in GCC protein levels and therefore indicating a relatively long half-life of this receptor in T84 cells (Fig. 3). This decrease in GCC protein may seem of relatively minor importance in T84 cells, in which GCC levels are extremely high. It is conceivable that in cells in which GCC expression is lower, such as the colonic crypt cells in the intestine [42], a reduction in mRNA levels of GCC could result in a more dramatic effect on lowering intracellular cGMP levels on guanylin stimulation.

Protein kinase C is known to regulate the transcription of a number of genes. AP-1 was identified as a transcription factor that bound to *cis*-elements in the promoter of phorbol ester-inducible genes, such as the human metallothionein IIa, collagenase, stromelysin and interleukin-2 [43]. The phorbol ester responsive element is a conserved palindromic sequence, ATGAG/CTCAG [43,44] recognized by a dimeric complex of jun or a heterodimer of fos and jun transcription factors [45–48]. The heterodimer of fos/jun forms the AP-1 complex, which is capable of transactivation. The other PKC-responsive *cis*-elements include the serum-responsive element of the *c-fos* gene [49,50], NF $\kappa$ B binding site [51,52] and the AP-2 recognition site [53]. However, these elements are recognized by transcription factors distinct from the AP-1 complex. Activation of PKC upon phorbol ester treatment leads to modulation by phosphorylation of components of the AP-1 complex that regulates their binding to the AP-1 site [43–53].

Regulation of transcription by PKC through the conventional AP-1/AP-2 regulatory sequences in the promoters of receptor guanylate cyclases other than GCC has been reported. Activation of PKC transcriptionally downregulates the natriuretic clearance receptor, NPR-C, through

multiple AP-1 sites present in the NPR-C promoter [54]. The presence of multiple AP-2 sites in the ROS-GC1 promoter [55] renders its transcription susceptible to modulation by PKC. Whereas PKC activation upregulates ROS-GC1 mRNA [55], NPR-C mRNA [56,57] is downregulated. However, there are no consensus AP-1 sites within the cloned 5' promoter of human GCC.

Interestingly, prolonged exposure of cells to ANP downregulates GCA mRNA, with increases in cGMP levels in the cell being essential to bring about the decrease in transcription of the mRNA [58]. This event presumably occurs through the activation of protein kinase G, and the cGMP-responsive element is located at a site in the GCA promoter > 1 kb upstream of the transcription start site. Results from our laboratory do not show any change in GCC mRNA levels in T84 cells on prolonged ST treatment under conditions in which GCC desensitization is observed [17], and no changes in phosphorylation of GCC are detected. Indeed, upon prolonged exposure of T84 cells to STh, the activation of a cGMP-binding, cGMP-specific phosphodiesterase, PDE5, accounts for a large reduction in cGMP levels, thereby inducing cellular refractoriness to the ST peptide [16,18].

Based on the significant reduction of [–129/+117] promoter activity on PMA treatment of T84 cells, we examined the possibility of modulation of HNF4 DNA binding activity upon PMA activation (Fig. 7). HNF4, a member of the superfamily of ligand-dependent transcription factors that includes the steroid hormone receptors, regulates the transcription of genes in the liver, kidney, intestine and pancreas [59,60]. PMA-mediated activation of PKC led to a loss of binding of HNF4 to its target site in the –50/–20 region of the GCC promoter (Fig. 7). Possible mechanisms of regulation of DNA-binding activity of HNF4 by PKC include lowered expression of HNF4 itself and/or direct phosphorylation of HNF4. There are reports that the DNA-binding activity of HNF4 is reduced upon hyperphosphorylation by PKA [61]. A putative PKC-dependent phosphorylation site could overlap the PKA-dependent phosphorylation site in HNF4, which lies within a region critical for DNA binding [62]. Another probable mechanism for regulating HNF4 binding to its sequence in the GCC promoter could be that a PKC-mediated phosphorylation event affects its association with additional transcription factors. However, little is known about the mechanisms of regulation of HNF4 by PKC; these possibilities need to be tested and are currently being investigated. It is very likely that under physiological conditions, regulation of GCC expression is the outcome of a concerted action among many transcription factors whose binding activity may be differentially regulated by PKC.

What is the physiological role of PKC-mediated modulation of GCC transcription? Carbachol treatment of T84 cells increases the ligand-induced activity of GCC via activation of PKC [24]. This suggests that activation of muscarinic receptors, or perhaps receptor tyrosine kinases, both of which have been shown to modulate PKC activity [63], could result in increased GCC activity, later coupled with a downregulation of GCC mRNA. Both guanylin and uroguanylin genes have multiple AP-1/AP-2 binding sites [64–66], suggesting that expression of the ligands for GCC may also be modulated by PKC activation. In addition, the promoter of the guanylin gene also has binding sites for

hepatic nuclear factor 1 (HNF1) which are important in regulating transcription of guanylin mRNA [66,67]. Transcription of HNF1 itself is, in turn, regulated by HNF4 [68]. In the context of chloride secretion from the intestinal cell, interesting speculations can also be drawn when one considers that CFTR mRNA is transcriptionally downregulated by PKC [56]. Treatment of T84 cells with PMA led to a reduced ability of these cells to upregulate chloride secretion through CFTR in a normal fashion upon forskolin treatment, via the well-established regulation of CFTR by cAMP. Therefore, it can be hypothesized that a signaling pathway that increases PKC activity can ultimately lead to a decrease in chloride secretion by downregulating a number of components that control chloride secretion from the cell. However, demonstration of this possible modulation of ligand, receptor and CFTR expression in the GCC signaling system, by a single regulatory molecule such as PKC or HNF4, remains to be made.

The results of this study clearly demonstrate the transcriptional regulation of GCC by PKC. Our observations may have both important implications for understanding GCC expression and its regulation by cross-talk with additional signaling pathways, and may also provide important inputs into a greater understanding of CFTR function and physiological role of the guanylin family of peptides in normal physiology. Further analysis of the GCC promoter for hitherto unknown regulatory factors would be essential to achieve a better understanding of the role and mechanisms of transcriptional regulation of GCC in both intestinal and extra-intestinal tissue.

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