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Expression and Regulation of the cGMP-Binding, cGMP-Specific Phosphodiesterase (PDE5) in Human Colonic Epithelial Cells: Role in the Induction of Cellular Refractoriness to the Heat-Stable Enterotoxin Peptide

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Abstract Stable toxin (ST) peptides are the causative agents for a severe form of watery diarrhea. These peptides bind to a membrane-associated form of guanylyl cyclase, guanylyl cyclase C. The result is an accumulation of cyclic guanosine monophosphate (cGMP) in the intestinal cell, regulating protein kinase activity and the phosphorylation of a number of proteins involved in ion transport across the intestine. Using the human T84 colonic cell line as a model system, we show that cGMP accumulation in these cells after ST application is regulated by the activity of the cGMP-binding, cGMP-specific phosphodiesterase (PDE5). The presence of human PDE5 in this cell line was confirmed by Western blot analysis, using an antibody raised to the bovine enzyme, and by the observation that cGMP hydrolytic activity detected in T84 cell lysates was almost completely inhibited by low concentrations of zaprinast, a specific inhibitor of PDE5. An increase in activity of PDE5 was observed in T84 cell lysates on exposure to the ST peptide and prolonged exposure of T84 cells to the ST peptide led to the induction of cellular refractoriness in these cells, which was largely contributed in terms of an increased rate of degradation of cGMP in desensitized cells as a result of PDE5 activation. This activation was correlated with an increase in the affinity of the enzyme for the substrate cGMP, as well as an increased affinity for zaprinast. We provide evidence for the first time that cGMP levels in the human colonocyte are regulated by the cGMP-hydrolytic activity of PDE5 and suggest that the expression and regulation of PDE5 in the intestine could therefore be important in controlling cGMP-mediated signaling in this tissue. J. Cell. Biochem. 77:159–167, 2000.

Key words: cyclic nucleotide phosphodiesterase; stable toxin; cGMP; T84 cells; guanylyl cyclase C

The heat-stable enterotoxins (ST) are a major cause of transitory diarrhea in travelers and young children in the developing world [Giannella, 1981]. Stable toxins are small cysteine-rich peptides [Arita et al., 1986; Aimoto et al., 1982; Takao et al., 1982], which mediate their action by binding to a member of the receptor guanylyl cyclase family, guanylyl cyclase C (GCC) [Schulz et al., 1990; Singh et al., 1991; De Sauvage et al., 1991; Garbers, 1992]. GCC also serves as a receptor for the newly discovered gastrointestinal hormones, guanylin and uroguanylin [Currie et al., 1992; Hamra et al., 1993]. Binding of these peptides to GCC leads to an increase in intracellular cyclic guanosine monophosphate (cGMP) levels, activation of an intestinal cell-specific cGMP-dependent protein kinase, and phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) leading to enhanced chloride ion transport and water efflux from the intestinal cell

Abbreviations used: CFTR, cystic fibrosis transmembrane conductance regulator; ELISA, enzyme-linked immunosorbent assay; GCC, guanylyl cyclase C receptor; GST, glutathione S-transferase; GST-PDE5, GST-fusion protein with PDE5; IBMX, isobutylmethylxanthine; PDE, cyclic nucleotide phosphodiesterase; PDE4, cAMP-specific PDE; PDE5, cyclic GMP-binding, cGMP-specific phosphodiesterase; ST, heat-stable enterotoxin; STh, ST peptide produced by a human isolate of enterotoxigenic *Escherichia coli*.

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[Field et al., 1978; Chao et al., 1994; Vaandrager et al., 1997]. While a reasonable amount of information is available related to the molecular characterization of GCC and its mode of activation by ST, very few studies address the signaling mechanisms that operate in the intestinal cell as a result of ST exposure.

We and others have been using the human colonic T84 cell line as a model system to study ST-mediated signal transduction events [Guarino et al., 1987; Visweswariah et al., 1992, 1994]. These cells were originally derived from a human colonic carcinoma [Dharmsathaphorn et al., 1984], have most of the properties of the human intestine such as showing vectorial ion transport and development of tight junctions, and were shown to express GCC in high amounts [Visweswariah et al., 1994]. Application of ST peptide to monolayer cultures leads to a dramatic increase in cGMP accumulation and enhanced chloride efflux. We have extensively characterized GCC from these cells.

ST-mediated diarrheas are transitory in nature and less severe than cholera toxin-mediated diarrheas [Giannella, 1981]. We therefore wished to study whether certain cellular mechanisms could control the duration and severity of ST-mediated diarrhea and hypothesized that cyclic nucleotide phosphodiesterases could permit the degradation of the second messenger, cGMP and thereby regulate downstream signaling. In studies reported recently, we have shown that prolonged exposure of T84 cells to ST leads to the induction of cellular refractoriness in terms of the ability of the desensitized cells to respond to fresh application of the toxin, as monitored by cGMP accumulation [Bakre et al., 1997]. We also demonstrated that while GCC desensitization was observed, in that the receptor showed a reduced ability to respond to ST when isolated from desensitized cells, the major cause of the induction of cellular refractoriness was the increased activity of phosphodiesterases, specifically, the cGMP-binding, cGMP-specific phosphodiesterase (PDE5) [Bakre et al., 1997].

Cyclic nucleotide phosphodiesterases (PDE) are a complex family of enzymes, some showing specificity for either cAMP or cGMP and some being dual specific [Conti et al., 1995; Beavo, 1995]. The availability of specific inhibitors for the various enzymes as well as antibodies and cDNA probes has allowed investigators to identify the specific PDE involved in the regulation of cyclic nucleotide accumulation in specific cell types. In many cases, phosphorylation and the synthesis of alternate transcripts of the enzyme regulate the activity of PDE isoforms, which control accumulation of the cyclic nucleotides in the cell [Beltman et al., 1993]. While there is a great deal of information suggesting the role of the cAMP-specific PDE (PDE4) in the induction of cellular refractoriness [Conti et al., 1983, 1995], there is virtually no information on the role of PDE regulating cGMP accumulation.

Our studies on the induction of cellular refractoriness to ST peptides in T84 cells showed that PDE5 was responsible for the increased degradation of cGMP [Bakre et al., 1997]. PDE5 has been characterized extensively, with the most information being available on the bovine enzyme. PDE5 has a low K_m for cGMP and can hydrolyze cAMP poorly [Francis et al., 1988]. The enzyme is active as a homodimer of two polypeptide chains of M_r 90kDa [Francis et al., 1994; Turko et al., 1998a; McAllister-Lucas et al., 1993, 1995]. Each chain contains a C-terminal catalytic core, which is conserved among all PDE. The N-terminal regulatory domain of the enzyme possesses two noncatalytic, high-affinity cGMP sites, but binding of cGMP to these sites does not appear to be prerequisite for catalytic activity. The enzyme can be phosphorylated in the N-terminal region, but cGMP binding is essential for phosphorylation to occur [Turko et al., 1998b]. PDE5 is expressed in high levels in rat aorta and lung and in the human small intestine, colon, and lung, as detected by Northern blot analysis [Kotera et al., 1997; Stacey et al., 1998]. Specific inhibitors for the enzyme are available (e.g., zaprinast and sildenafil citrate), which help in its characterization and identification. The role of PDE5 in vascular smooth muscle cells is to regulate cGMP accumulation that occurs on NO production or stimulation by the atrial natriuretic peptides [Moreland et al., 1998]. Our earlier studies had indicated an important role for PDE5 in human colonic epithelial cells [Bakre et al., 1997]. In this report, we have generated an antibody to PDE5 in order to examine expression of the enzyme and the mechanism by which PDE5 is activated in T84 cells on the addition of ST. Our results suggest the possibility of a feedback loop, which regulates cGMP accumulation in human colonic cells via its more rapid degradation after initial accumulation.

MATERIALS AND METHODS

All restriction enzymes were obtained from Boehringer-Mannheim (Germany) and Bangalore Genei (Bangalore, India). Dulbecco's modified Eagle's medium (DMEM):F12, phosphate-free DMEM medium, newborn calf serum, and adjuvants were from Life Technologies (USA). The plasmid vectors were from Pharmacia (Sweden). STh was purified from *Escherichia coli*-hyperexpressing strains [Dwarkanath et al, 1987]. ^{3}H-cGMP (spec act 14.8Ci/mmol) was obtained from Amersham Life Science (USA).

Culture and Maintenance of T84 Cells

T84 cells obtained from ATCC (CCL 247), were maintained in DMEM:F12 containing 5% newborn calf serum, penicillin, and streptomycin as described earlier [Visweswariah et al., 1992]. Cells were used at approximately 75–90% confluency, after 4–7 days in culture.

ST-Mediated Accumulation of cGMP in T84 Cells

T84 cells cultured in 24-well dishes were preincubated in medium alone, or in the presence or absence of IBMX (500 μ M) for 30 min at 37°C before stimulation with STh (10^{-7} M) for various periods of time. Cells were washed with warm, serum-free medium, lysed in 0.1 M citric acid and cGMP in the lysates measured by radioimmunoassay (RIA) [Visweswariah et al., 1994].

In Vitro Phosphodiesterase Assays

Cells were grown to confluence and incubated without or with STh (10^{-7} M), for varying periods of time in serum-free DMEM:F12 at 37°C in a 5% CO_2 humidified incubator. Cells were washed with chilled phosphate buffer (10 mM sodium phosphate, pH 7.5 containing 0.9% NaCl; phosphate-buffered saline [PBS]) and were harvested in homogenization buffer (50 mM Tris-HCl, pH 7.5 containing 1 mM benzamidine, 2 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 5 mM β-mercaptoethanol, 10 nM okadaic acid, and 100 μM sodium orthovanadate). The cell lysate was sonicated briefly and centrifuged for 1 h at 10,000*g* at 4°C. Supernatant was used as the source of enzyme for the in vitro PDE assays. The assay was performed essentially as described earlier [Bakre et al., 1997], with some modifications. Assay mixtures contained 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, 0.3 mg/ml bovine serum albumin (BSA), and varying concentrations of cGMP and ^{3}H-cGMP. cGMP concentration was varied from 50 μ M to 0.1 μ M. The assay was initiated by the addition of the enzyme. In addition to the samples to be assayed, enzyme blanks containing heat-inactivated enzyme or no enzyme at all, were included. Assay mixtures were incubated at 30°C for 15 min, and the reaction terminated by placing the assay tubes for 2 min in a boiling water bath. After cooling the samples, 50 μg of *Crotalux atrox* snake venom 5′-nucleotidase was added to each tube and incubation continued at 30°C for 30 min. In order to separate unreacted ^{3}H-cGMP, and cGMP from ^{3}H-guanosine, each reaction mixture was chromatographed on DEAE-Sephadex A-25 columns (0.7 × 8 cm; Pharmacia) equilibrated in 20 mM ammonium formate, pH 7.5, at 25°C. Columns were washed with 3 ml of 20 mM ammonium formate; radioactivity in the washes was monitored in aqueous scintillation cocktail.

Construction of Plasmid pGEX-PDE5a and Expression of Glutathione S-Transferase Fusion Protein (GST-PDE5)

The cDNA for bovine PDE5 was a kind gift from Dr. J.D. Corbin, Vanderbilt University (Nashville, TN) [McAllister-Lucas et al., 1993]. Primers were designed to amplify the N-terminal unique domain of PDE5 consisting of 420 base pairs (bp). The 5′ primer with a sequence 5′ GCGCTGACCATGGAGAGGGCCG3′ corresponded to sequences in the N-terminal region of the PDE5 clone and included a *Nco*I restriction site. The 3′ primer with a sequence 5′-ATTCCAAGGATCCCTAGCACT-3′ was to the end of unique domain and contained a *Bam*HI site followed by a stop codon. Using the full-length cDNA as the template, polymerase chain reaction (PCR) was performed using Pfu polymerase (Stratagene), and the following PCR profile was used: 94°C, 45 s; 55°C, 45 s; and 72°C, 2 min. The PCR-amplified product was ligated into *Sma*I cut pGEX-5X-2 to generate pGEX-PDE5a, and the clone obtained was sequenced to confirm that no mutations had been generated during the PCR reaction.

E. coli (DH5α) cells harboring the plasmid pGEX-PDE5a were grown in Luria-Bertani broth at 37°C. At an A_{600} of 0.5, the cells were induced with 1 mM isopropyl β-thiogalactopyranoside for 4–5 h at 37°C. The cells were harvested by centrifugation and washed 3 times

with PBS. Cells were lysed by sonication in buffer containing 50 mM Tris-HCl, pH 8.0. 5 mM dithiothreitol (DTT), 0.1 M NaCl, 1 mM benzamidine, 5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and 1% Triton X-100 (Virtis 475 sonicator). The cell lysate was fractionated by centrifugation at 12,000*g* for 15 min at 4°C, and the supernatant fraction was loaded onto glutathione-coupled, epoxy-activated Sepharose 6B beads affinity matrix (Pharmacia). After the adsorption to the beads at 4°C for 16 h, the resin was washed in buffer and bound protein was eluted with 5 mM reduced glutathione. The cytosolic fraction as well as the pure protein eluted was analyzed on a 12% SDS-polyacrylamide gel [Laemmli, 1970].

Generation of Polyclonal Antibodies to GST-PDE5a

Pure GST-PDE5a fusion protein was used to raise polyclonal antibodies in rabbits. The primary dose of immunogen (500 µg) was in Freund's complete adjuvant, and animals were boosted with 250 µg of fusion protein in Freund's incomplete adjuvant and the presence of antibody detected by ELISA and Western Blot analysis using immobilized fusion protein (data not shown). The IgG fraction was prepared from the serum by ammonium sulfate precipitation.

Immunodetection of PDE5

Cytosolic protein from T84 cells and partially purified bovine PDE5 (gift of Dr. J. Corbin, Vanderbilt University) were subjected to sodium dodecyl sulfate (SDS) gel electrophoresis in 10% polyacrylamide gel, and the proteins were transferred to nitrocellulose membrane (Hybond enhanced chemiluminescence [ECL], Amersham) in 25 mM Tris, 190 mM glycine buffer, containing 20% methanol. Transfer was carried out for 1.5 h at 25°C. The membrane was blocked using 5% blocking agent (Amersham) for 1 h, after which the membrane was washed in PBS containing 0.1% Tween 20. Membrane was incubated with either preimmune IgG or GST-PDE IgG (stock: 12 mg/ml) diluted 1:10,000 in PBS containing 0.2% BSA and 0.1% Tween 20 for 2 h at 25°C. Membrane was washed in PBS containing 0.1% Tween-20 and was further incubated with anti-rabbit horseradish peroxide (HRP) conjugate (Amersham;1: 2,500) diluted in PBS containing 0.2% BSA and 0.1% Tween 20) for 1 h. The presence of antibody was detected by ECL, using the ECL Plus kit (Amersham) according to manufacturer's instructions.

Ion-Exchange Purification of PDE5 From T84 Cells

The purification procedure was an adaptation of the method described earlier [Francis et al., 1988]. Confluent monolayers of T84 cells grown in 6-cm dishes were incubated in medium alone or in the presence of STh (3×10^{-7} M) for 18 h in serum-free DMEM: F12 at 37°C in a 5% CO_2 humidified incubator. Cells were washed in the same medium and harvested in ice-cold buffer containing 20 mM sodium phosphate buffer, pH 6.8, 2 mM EDTA, and 25 mM β-mercaptoethanol. Cells were briefly sonicated and centrifuged at 10,000*g* for 1 h at 4°C. The supernatant obtained was subjected to anion exchange chromatography by applying the protein to a DEAE-Sephacel column (bed volume 2 ml) equilibrated with cell lysis buffer. The column was washed with 10 bed volumes of lysis buffer and PDE5 specifically eluted using buffer containing 0.25 M NaCl. Fractions (500 µl) were collected and checked for phosphodiesterase activity as described above. Protein was estimated by the method of Bradford [1976].

Data Representation and Statistical Evaluation

All data were evaluated by GraphPad Prism (GraphPad Software., San Diego, CA).

RESULTS

Activation of PDE5 in T84 Cells Exposed to the ST Peptide

Our earlier studies had indicated that prolonged treatment of T84 cells with the ST peptides resulted in cellular refractoriness to fresh stimulation with the ST, as a result of receptor desensitization as well as PDE5 activation [Bakre et al., 1997]. We monitored cGMP accumulation in whole cells on exposure to ST for various periods of time both in the presence and in the absence of the general phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX). The data shown in Figure 1A indicate that a significant increase in cGMP accumulation in T84 cells was observed when cells were treated with IBMX before ST application. Moreover, the increase in cGMP levels observed in the presence

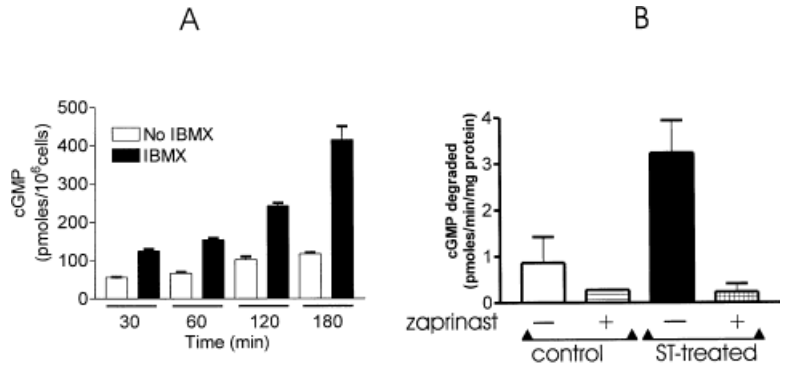

Fig. 1. Role of PDE5 in regulating cGMP levels in T84 cells. **A:** T84 cells were stimulated with STh (10^{-7} M) for the indicated periods of times in the presence and the absence of IBMX. Cells were lysed and cGMP levels measured in cell lysates by radioimmunoassay. Values represent the mean±SEM of experiments performed 3 times with duplicate determinations at each time point. **B:** Cell lysates were prepared from T84 cells that had been treated with STh for 3 h and phosphodiesterase assays performed using cGMP as the substrate. Assays were also performed in the presence of 10 μM zaprinast. Values represent the mean±SEM of assays performed in triplicate with experiments repeated twice. All values obtained in the presence of IBMX were significantly higher than that in the absence of IBMX ($P < 0.05$) when analyzed by the Student's *t*-test.

of IBMX was more pronounced in cells that had been treated for 3 h with the ST peptide, indicating that phosphodiesterase activity was apparently increased after ST application. We therefore measured cGMP-hydrolyzing activity in lysates prepared from T84 cells after treatment with ST for 3 h; the data shown in Figure 1B indicate that an increase in PDE activity was observed 3 h after the addition of ST. This increased activity was almost completely inhibited by zaprinast, indicating that PDE5 was largely responsible for the degradation of cGMP after ST application.

Generation of a PDE5-Specific Antibody and Western Blot Analysis With T84 Cell Lysates

The human isoform of PDE5 has been recently cloned and is predicted to have a high degree of sequence homology to the earlier characterized rat and bovine enzyme [McAllister-Lucas et al., 1993; Kotera et al., 1997; Stacey et al., 1998; Loughney et al., 1998]. Significant homology is also observed with other PDEs in the catalytic domain of the enzyme [Stacey et al., 1998]. We therefore expressed a fragment of the bovine enzyme as a GST-fusion protein, and the region that we chose comprises the N-terminus of the enzyme, which is a sequence present only in PDE5. We should therefore generate an antibody specific for PDE5. Figure 2A shows a gel picture of the GST-PDE fusion protein, as expressed in *E. coli* cells and after purification of the enzyme using glutathione-agarose affinity chromatography. The lower major band is a degradation of the fusion protein

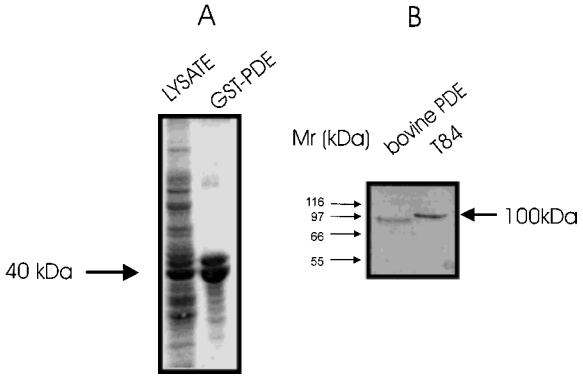

Fig. 2. Expression of GST-PDE5 and generation of antibodies to native PDE5. **A:** *Escherichia coli* cells transformed with GST-PDE5 plasmid were induced and lysates prepared. GST-PDE5 from the cytoplasm of cells was purified by glutathione affinity chromatography to obtain a 40-kDa protein. **B:** Western blot analysis using antibodies raised to GST-PDE5. Partially purified bovine PDE5 and cytoplasm prepared from T84 cells (20 μg) was loaded on a 10% SDS gel and proteins transferred to PVDF membrane for Western blot analysis with polyclonal antibodies to GST-PDE5. Bound antibody was detected using ECL.

as monitored by Western Blot analysis, using an antibody to GST (data not shown). Purified protein was used to generate antibodies in rabbits and Figure 2B shows Western blot analysis performed with a partially purified preparation of PDE5 expressed in baculovirus as well as lysates prepared from T84 cells. A band of 100,000 Da is observed in T84 cells, which corresponds in size to that predicted from the human PDE5 cDNA clone described earlier [Stacey et al., 1998; Loughney et al., 1998].

Increased Activity of PDE5 Observed on ST Treatment Is Not Due to an Increase in PDE5 Content in the Cells

We measured cGMP hydrolytic activity in T84 cells as a function of time of ST treatment. As shown in Figure 3, a dramatic increase in PDE activity was observed at 3 h, followed by a gradual decline to 9 h. We analyzed samples prepared from T84 cell lysates at various times by Western blot analysis, using the antibody that we had raised to PDE5. As shown in Figure 3 (inset), there did not appear to be a dramatic change in the amount of immunoreactive PDE5 during the time that we had observed increased activity. This indicates that overall changes in PDE5 content in T84 cells were not responsible for the increased catalytic activity of the enzyme observed on ST treatment.

Activation of PDE5 as a Means of Inducing Cellular Refractoriness in T84 Cells

Earlier results had provided evidence that prolonged exposure (>18 h) of T84 cells to the ST peptide led to the induction of cellular refractoriness as reflected in an increased rate of degradation of cGMP in ST-treated cells. To investigate the reason for the change in the catalytic activity of PDE5, we prepared lysates from control and T84 cells treated for 18 h with ST and performed cGMP PDE assays. As shown in Figure 4, increased PDE activity was observed, in agreement with our earlier reported results, and activity in lysates prepared from

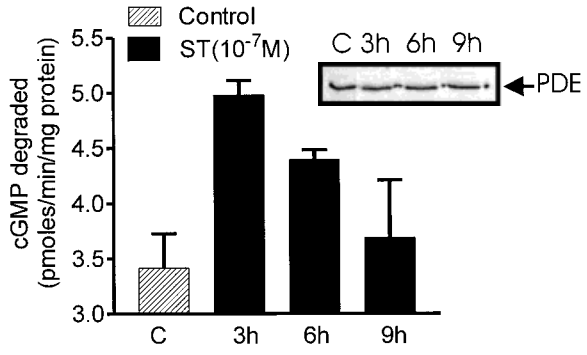

Fig. 3. Time course of activation of PDE5 in T84 cells after STh treatment. STh (10^{-7} M) was applied to T84 cells and lysates prepared for PDE assays. Values represent the mean±SEM of assays performed 3 times, with duplicate determinations for PDE activity. **Inset:** a Western blot performed with GST-PDE5 antiserum and protein (20 μg) from lysates prepared at different time points. Data are representative of experiments performed at least 3 times.

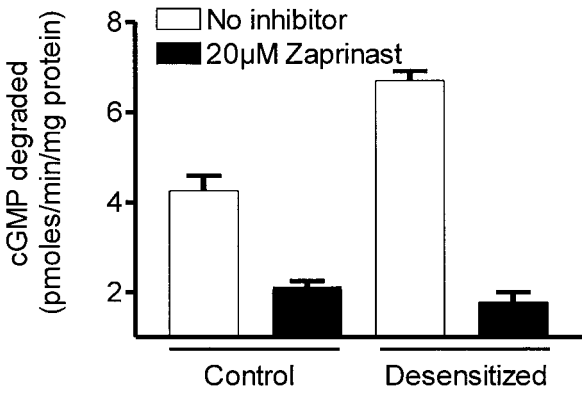

Fig. 4. Increased activity of PDE5 observed on partial purification from cytosol of ST-treated cells. Cytosol was prepared from T84 cells with or without treatment with ST (10^{-7} M) for 18 h, and subjected to partial purification as described under Materials and Methods. Fractions were tested for PDE activity in the absence and presence of zaprinast (10 μ M). Values represent the mean±SEM of duplicate determinations with experiments performed 3 times.

both control and ST-treated cells was significantly inhibited by zaprinast, indicating that the major contribution to cGMP hydrolytic activity was from PDE5. In an effort to determine whether the increased activity observed at 18 h could be detected in partially purified fractions of PDE5, we subjected the control and the ST-treated cell lysates to a single step of ion exchange chromatography, normalized the amount of PDE5 assayed by Western blot analysis, and performed PDE assays with varying concentrations of cGMP. The data shown in Figure 5 indicate that there was a slight, but significant change in the K_m value of the enzyme for the substrate cGMP. The K_m value of the enzyme from desensitized cells was four-fold lower than that in control cells. However, a decrease in the V_{max} of the enzyme was also observed. When we monitored inhibition of enzyme activity in the presence of varying concentrations of zaprinast, the enzyme prepared from desensitized cells was found to be inhibited at a lower concentration of zaprinast (Table I). It appears that the increased activity of PDE caused by the reduction in the K_m value of the enzyme for the substrate could make it more efficient in hydrolyzing low concentrations of cGMP, which are produced on initial restimulation of the cells. This perhaps contributes to the cellular refractoriness that is observed in T84 cells on prolonged ST treatment, along with additional factors such as receptor desensitization. To our knowledge, this is the first report on the change

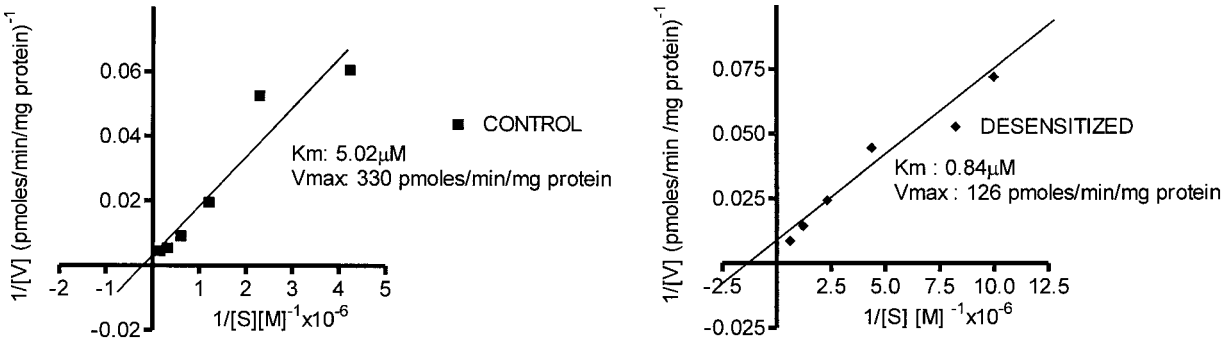

Fig. 5. Kinetic properties of partially purified PDE5 from control and desensitized T84 cells. A double reciprocal plot of the data obtained from a Michaelis-Menten plot of cGMP-hydrolyzing activity of partially purified PDE5 prepared from control and desensitized T84 cells, using varying concentrations of cGMP (0.1 μ M-20 μ M). Values shown are representative of data obtained from two individual experiments.

TABLE I. Sensitivity of PDE5 to Zaprinast From Control and Desensitized Cells*

T84 cells	IC_{50} for Zaprinast (μM)
Control	6.65 ± 0.54
Desensitized	1.51 ± 0.29

*Monolayers of T84 cells were incubated in serum-free medium either in the absence or presence of STh (3×10^{-7} M) for 18 h. Cell extracts prepared from control and desensitized cells were used to perform in vitro PDE assays. IC_{50} values for zaprinast were assessed at 1 μM cGMP. Values represent the mean±SEM of experiments performed at least 3 times on three different preparations of control and desensitized cytoplasm.

in K_m of the PDE5 during a cellular response, and appears to be the first data to indicate the importance of PDE5 in regulating cGMP levels in colonic cells.

DISCUSSION

This report provides the first evidence for the role of a PDE5 in desensitizing cells to a stimulus that elevates cGMP in a cell. Elegant studies have shown much earlier that PDE4, the cAMP-specific phosphodiesterase, had an important role to play in the induction of cellular refractoriness to follicle-stimulating hormone (FSH) in Sertoli cells [Conti et al., 1993]. FSH elevates cAMP levels in the cell; altered transcription of different splice variants of PDE4 were detected during the response of the cell to prolonged exposure to the hormone [Conti et al., 1982]. While there is evidence for the presence of at least two splice variants in human PDE5 [Stacey et al., 1998], it remains to be seen whether they have a role to play during the activation of PDE5 in T84 cells. In the case of PDE4, increased phosphorylation of the enzyme was also observed during short-term treatment of cells to the hormone and phosphorylation alone could increase the activity of the enzyme, in contrast to the results that we report here [Conti et al., 1982]. PDE4 does not have a noncatalytic cAMP-binding site, so mechanisms of regulation of individual enzymes may be markedly different.

It is unlikely that PDE5 undergoes phosphorylation without the presence of cGMP in the cell. Binding of cGMP to the noncatalytic sites of PDE5 is essential for its further phosphorylation by cGMP- or cAMP-dependent kinases [Turko et al., 1998b]. Experiments performed recently using rat vascular smooth muscle cells and stimulation after atrial natriuretic factor treatment provided evidence for the rapid phosphorylation of PDE5, correlated with an increase in the catalytic activity [Wyatt et al., 1998]. This is in contrast to the far more prolonged activation that we see in T84 cells. It is therefore possible that phosphorylation may not contribute to the activation of the enzyme in T84 cells, but this remains to be investigated. Perhaps the more prolonged effects of activation in colonic cells are an adaptation to the requirement for the induction of cellular refractoriness in this particular tissue.

A number of point mutations have been made in bovine PDE5 in the catalytic site, but none of them resulted in a change in the K_m of the mutant enzymes [Turko et al., 1998a]. Moreover, there was no change in the affinity of the enzyme for zaprinast as well, which is expected since zaprinast is a competitive inhibitor of the enzyme. Concentrations of cGMP used in ear-

lier assays may have been too high to detect any small change in affinity for the substrate, as we have seen in our experiments. While there is extensive sequence homology between the bovine PDE5 and the human enzyme, it is also possible that regulation of the two enzymes may differ and future studies along these lines would provide more information.

Interestingly, thus far no reports have described the role of phosphodiesterases in the intestine, despite the obvious importance of the action of cyclic nucleotides in this tissue. Evidence from Northern blot analysis has indicated good expression of PDE5 in the small intestine [Kotera et al., 1997; Stacey et al., 1998]. However, it is possible that the smooth muscle cells of the intestine largely contribute to the observed high-level expression. Our data reported in this article indicate that epithelial cells of the intestine harbor significant PDE5 activity. Expression of other components of the cGMP signal transduction machinery, such as GCC, cGMP-dependent protein kinase II, and CFTR, is seen in intestinal epithelial cells [Trezise et al., 1991; Jarchau et al., 1994; Nandi et al., 1997]. Our studies have shown that PDE5 is now to be included as an important component in the signal transduction cascade; we are currently attempting to localize PDE5 in various regions and cell types of the intestine.

An interesting point raised in our observations on the expression and importance of PDE5 in the intestine is related to the recent introduction of sildenafil citrate (Viagra™) as a treatment for erectile dysfunction [Moreland et al., 1998]. Sildenafil citrate is the most potent inhibitor known so far for PDE5 [Moreland et al., 1998; Corbin et al., 1999]. One of the side effects reported by individuals on sildenafil citrate was diarrhea, reported in as many as 15% of patients [Moreland et al., 1998]. Our results suggest that inhibition of PDE5 in intestinal epithelial cells could permit the accumulation of cGMP in cells, which in turn could trigger the activation of downstream events as seen in ST-mediated diarrhea. The discovery of guanylin and uroguanylin peptides as gastrointestinal hormones that bind to GCC and elevate cGMP [Forte et al., 1996] could explain why ingestion of sildenafil citrate by certain individuals could result in diarrhea. Studies designed to address these issues are under way in the laboratory and should emphasize the importance of the results presented in this study.

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