Atrial natriuretic peptide modulates cystic fibrosis transmembrane conductance regulator chloride channel expression in rat proximal colon and human intestinal epithelial cells

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Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR) is one of the most intensively investigated Cl^- channels. Different mutations in the CFTR gene cause the disease cystic fibrosis (CF). CFTR is expressed in the apical membrane of various epithelial cells including the intestine. The major organ affected in CF patients is the lung, but it also causes an important dysfunction of intestinal ion transport. The modulation of CFTR mRNA expression by atrial natriuretic peptide (ANP) was investigated in rat proximal colon and in human intestinal CaCo-2 cells by RNase protection assay and semi-quantitative reverse transcriptase PCR techniques. Groups of rats subjected to volume expansion or intravenous infusion

of synthetic ANP showed respective increases of 60 and 50% of CFTR mRNA expression in proximal colon. CFTR mRNA was also increased in cells treated with ANP, reaching a maximum effect at 10^{-9} M ANP, probably via cGMP. ANP at 10^{-9} M was also able to stimulate both the CFTR promoter region (by luciferase assay) and protein expression in CaCo-2 cells (by Western blot and immunoprecipitation/phosphorylation). These results suggested the involvement of ANP, a hormone involved with extracellular volume, in the expression of CFTR in rat proximal colon and CaCo-2 intestinal cells.

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Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is the most intensively investigated Cl^- channel and it was the first anion channel to be identified by expression cloning (Riordan *et al.* 1989). CFTR gene emerged from the search for the cystic fibrosis (CF) locus (Nilius & Droogmans 2003). It belongs to a family of proteins called traffic ATPases or ATP-binding cassette (ABC) transporters (Nilius & Droogmans 2003). CF is characterized by reduction or abolishment of cell Cl^- secretion that leads to alterations in fluid and electrolyte epithelial transport causing abnormalities mainly of the respiratory and gastrointestinal systems (O'Loughlin *et al.* 1991, McCray *et al.* 1992).

Meconium ileus is observed in the gastrointestinal tract of approximately 10% of newborn CF patients, and obstructive gut disease is seen at later ages (Grubb & Boucher 1999). In addition, in CFTR-knockout mice a phenotype was found that closely resembles the phenotype

presented by CF patients, but the airway symptoms were not observed. In fact, most of these mice die from intestinal complications, which clearly indicates the crucial role of CFTR, especially in colonic Cl^- secretion, and the absence of effective compensation (Snouwaert *et al.* 1992, Eckman *et al.* 1995).

CFTR protein has been localized on apical membranes of epithelial cells in both small and large intestines (Crawford *et al.* 1991, Gaillard *et al.* 1994). Functional analysis of cAMP-activated Cl^- conductance and *in situ* hybridization studies showed the expression of CFTR in the intestinal cell population responsible for Cl^- secretion mainly in crypt cells, where CFTR is highly expressed (Strong *et al.* 1994).

The CFTR not only functions as a Cl^- channel, it can regulate other ion transporters such as outwardly rectifying chloride channels (ORCCs; Schwiebert *et al.* 1995) and the amiloride-sensitive epithelial sodium channel (ENaC; Stutts *et al.* 1995), and this regulation is also observed in the colon mucosal epithelium and/or colonocytes

in culture (Ecke *et al.* 1996, Mall *et al.* 1999). After stimulation of wild-type CFTR by cAMP, amiloride-sensitive Na^+ absorption is inhibited, while ORCCs are stimulated. For that reason, changes in the conductance and/or expression of CFTR can be relevant in NaCl transport and the consequent trans-epithelial fluid movement.

Hormonal modulation of ions transporters in the kidney and intestinal epithelia is well known to be important in extracellular volume regulation. Classical extracellular volume-regulating hormones such as aldosterone and glucocorticoids induce electrogenic absorption not only in the kidney but also in the distal colon (Grotjohann *et al.* 1999, Coric *et al.* 2004). The hormonal modulation of chloride channels in the apical membrane of epithelial cells was found in the kidney (Morales *et al.* 2001, Jentsch *et al.* 2002, Ornellas *et al.* 2002, Santos Ornellas *et al.* 2003), and also in the distal part of the intestine, particularly in the proximal and distal colon (Schroeder *et al.* 2000, Estevez *et al.* 2001, Kunzelmann & Mall 2002).

Atrial natriuretic peptide (ANP) is a 28-amino acid polypeptide hormone secreted mainly by the heart atria in response to atrial stretch and it is produced by changes in blood volume and/or arterial pressure (Dietz 1984, 1987). ANP was found to be synthesized in stomach and small and large intestine of humans and rats (Vuolteenaho *et al.* 1988, Ehrenreich *et al.* 1989, Gonzalez Bosc *et al.* 2000). This peptide is a classical inhibitor of Na^+ reabsorption, mainly in the cells of the renal collecting ducts (Maack 1996), but in the gastrointestinal tract it has an inhibitory effect on mineralocorticoid receptors in the proximal colon, inhibiting the Na^+ absorption induced by aldosterone (Schulman *et al.* 1996), probably via cGMP and protein kinase G intracellular second messengers (Argenzio & Armstrong 1993, Waldman *et al.* 1984). Furthermore, it is known that the conductance of Cl^- channels is effectively regulated by protein kinase G (Lin *et al.* 1992). Thus, it is reasonable to postulate that ANP can act on epithelial cells through its cyclase-coupled receptor and, thereby, modulates Cl^- channel conductance and/or expression.

The main purpose of this work was to increase our knowledge of CFTR chloride channel expression control in the gastrointestinal tract by studying its expression regulation in proximal colon of rats subjected to extracellular volume expansion or intravenous infusion of synthetic ANP. CFTR channel expression modulation by ANP was also investigated in human colon adenocarcinoma cells (CaCo-2 cells).

Materials and Methods

Cell culture

Human CaCo-2 cells were chosen for the present study because of their morphologic and functional characteristics, similar to normal human enterocyte cells, when

reaching confluence in culture (Grasset *et al.* 1985). CaCo-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco BRL), 25 mM glucose, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL) at 37 °C in a fully humidified atmosphere of 5% CO_2 in air. CaCo-2 cells were grown until they reached 90% of confluence, and then incubated with DMEM in the absence of fetal bovine serum for 12 h, and treated with ANP (10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} and 10^{-6} M) in DMEM without fetal bovine serum, at 37 °C for 24 h on 35 mm tissue-culture dishes. As we shown in previous reports after 24 h we can see changes in mRNA expression in cells treated with different hormones (Morales *et al.* 2001, Santos Ornellas *et al.* 2003).

The ANP hormone was diluted in double-distilled water. To study ANP action via a secondary signaling pathway on CFTR mRNA expression, cells were incubated with 1 mM cGMP, 5 µM cGMP inhibitor (cGMPI; Chang *et al.* 1991, Sood *et al.* 1992) and 10^{-9} M ANP, divided into different groups: A, non-treated cells (NT); B, cells treated with ANP; C, cells treated with ANP+cGMPI; D, cells treated with cGMP+cGMPI; E, cells treated with cGMP; F, cells treated with cGMPI; and G, cells treated with ANP+cGMP. Cells incubated only with DMEM without bovine fetal serum during 24 h were used and called the control NT group.

Animal preparation

The Ethics Committee of the Biophysics Institute, Federal University of Rio de Janeiro, previously approved all procedures and protocols using animals mentioned in this paper. Male adult Wistar rats (weight of 300–350 g) were kept in a room with controlled lighting (12 h light:12 h darkness; lights on at 08:00 h, off at 20:00 h) and temperature (23–27 °C). All groups had free access to food and tap water until the time of the experiment.

Rats subjected to volume expansion or continue i.v. infusions were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and 5 mg of diazepam, and then the femoral vein was catheterized for extracellular-expansion and ANP infusion-experiments. Rats subjected to volume expansion were infused intravenously with 10 ml of isotonic saline (0.9%) for 5 min (2 ml/min; Kato *et al.* 1986), and 1 h after the beginning of injection the rats were killed and proximal colon samples removed.

In the group of rats subjected to continuous i.v. infusion, synthetic ANP (α -atriopeptin, or rANP; Sigma) in 0.9% NaCl was infused as a 4 µg/kg priming dose over a 2 min interval, followed by continuous i.v. infusion at 0.5 µg/kg per min (1.56 ml/hour) for 60 min. After this procedure the animals were killed and the proximal colons removed. Control-group rats were infused intravenously with 0.9% NaCl (at 1.56 ml/hour; Dunn *et al.* 1986).

Abdominal aorta and vena cava were sectioned in order to kill the animals. This procedure leads to a massive hemorrhage that quickly kills the animals. Then proximal colon samples were removed, rinsed with PBS solution and total RNA immediately extracted from tissues using Trizol® reagent (Gibco BRL).

Isolation of total RNA

Total RNA was extracted from CaCo-2 cell cultures and rat proximal colon tissues using Trizol® reagent, following the protocol suggested by the manufacturer. The isolated RNAs were treated with 1 U/μl RNase-free DNase I (Gibco BRL) for 30 min to eliminate contamination with genomic DNA. Autoclaved diethylpyrocarbonate-treated water was used to dissolve the RNA, which was quantified by spectrophotometric absorbance at 260 nm.

RNase protection assay (RPA)

A human CFTR 247 bp segment (nucleotides 531–778) obtained through reverse transcriptase PCR (RT-PCR) was subcloned into pCR-Script SK(+). One microgram of this plasmid was linearized using NotI restriction enzyme. Following the Maxiscript kit protocol (Ambion, Austin, TX, USA) templates were transcribed *in vitro* in the presence of T7 RNA polymerase, [α -³²P]UTP (3000 Ci/mmol; NEN-Dupont, Boston, MA, USA) and nucleotides guanine, adenosine, thiamine and cytosine, generating radiolabelled antisense RNA probes. All probes were evaluated with 10 μg yeast tRNA in the presence and absence of RNase A and T₁ following the RPAII kit protocol (Ambion). Total RNA (30 μg) from CaCo-2 cells was mixed with radiolabelled antisense probe to CFTR (1×10^5 c.p.m./sample) and pTRI-β-actin-125-human (1×10^5 c.p.m./sample; Ambion), a human β-actin radiolabelled antisense probe to actin (127 bp) corresponding to nucleotides 853–979 of β-actin human sequence (an internal control), and hybridized at 45 °C for 18 h. Previous experiments were made to set optimal RNA quantities. These products were then treated with a mixture of RNase A and T₁ at 37 °C for 30 min. The protected fragments were separated by electrophoresis on 8 M urea gels containing 50 g/l polyacrylamide. The samples were transferred to chromatography paper and exposed to X-ray films with an intensifying screen at –70 °C for 24 h. After the films had been developed the density of the bands corresponding to the expected sizes of the probes was analyzed by Scion Image Alpha 4.0.3.2 software (Scion Corporation, Frederick, MD, USA).

RT-PCR

Total RNA (250 ng) was used to prepare first-strand cDNA. These total RNAs were first primed with

oligo(dT) primer and then reverse-transcribed with SuperScript™ (Gibco BRL) at 37 °C for 60 min. The negative control corresponded to a 250 ng aliquot of total RNA used for cDNA synthesis in the absence of the RT enzyme, and was called RT(–). The cDNA synthesis reaction was interrupted by DNA extraction using a mixture containing phenol, chloroform and isoamyl alcohol (PCI-25:24:1, vol/vol), and then precipitated with 100% ethanol and ammonium acetate (7.5 M) for 24 h at –20 °C. Finally, the cDNA was re-suspended in 5 μl of deionized water.

In the PCR reaction, cDNAs were used in the presence of 2.5 units Taq polymerase (Gibco BRL), 0.2 μM of each primer (CFTR and β-actin), 0.2 μM of each nucleotide and commercial buffer (Gibco BRL) containing 1.5 mM MgCl₂. PCR was performed under the following conditions: initial denaturation at 94 °C for 4 min, followed by 36 cycles of denaturation (94 °C, 1 min), annealing (58 °C, 1 min) and extension (72 °C, 1 min). The reaction was concluded with an extension for 10 min at 72 °C. Preliminary experiments were done to set the optimal conditions for the RT-PCR. The primers used in CFTR PCR reactions in CaCo-2 cells samples were 5'-CAC GCT TCA GGC ACG AAG-3' and 3'-GCA GAG GTG GCT GCT TC-5', which produced a DNA segment of 268 bp. As an internal control, human β-actin primers 5'-GTT GCT ATC CAG GCT GTG-3' and 3'-CAC TGT GTT GGC GTA CAG-5' were used, which produced a segment of 485 bp.

The primers used for the CFTR PCR reactions in rat proximal colon samples were 5'-CTG CCA TTG CTG GAG TTG-3' and 5'-GCA GCC ATC CTC TAG AAC-3', which produced a DNA segment of 615 bp. As an internal control the rat GAPDH primers 5'-GTC TTC ACC ACC ATG GAG-3' and 3'-CAT GAC AAC TTT GGC ATC-5' were also used, which produced a segment of 211 bp. For RT-PCR of natriuretic peptide receptors (NPRs) in CaCo-2 cells we used the following human primers: (i) human NPR type A (NPR-A) primers 5'-GCA TTG AGC TGA CAC GAA AA-3' and 5'-CCT TGA CGA TGT CAT TGG TG-3', which produced a segment of 219 bp, (ii) human NPR type B (NPR-B) primers 5'-AGC GCT GAA GAT CCA TGT CT-3' and 5'-GGA GTC CAG GAG GTC CTT TC-3', which produced a segment of 155 bp and (iii) human NPR type C (NPR-C) primers 5'-GGA GAC CGA TAT GGG GAT TT-3' and 5'-CAC TGC CGA TTC TTC TAG GC-3', which produced a segment of 222 bp.

The identities of amplified products was confirmed by determination of molecular size on agarose gel electrophoresis (1.5% agarose in buffer containing 40 mM Tris/acetate and 1 mM EDTA) and visualized by ethidium bromide staining (0.5 μg/ml) under ultraviolet light. The CFTR, β-actin and GAPDH bands from samples were analyzed by densitometry (Scion Image Alpha 4.0.3.2). Expression was normalized by dividing the CFTR values

by the corresponding internal control values (β -actin or GAPDH) amplified in the same reaction tube.

Immunoprecipitation and phosphorylation of CFTR

Biochemical analysis of CFTR expression and glycosylation was performed by immunoprecipitation with anti-CFTR antibodies followed by *in vitro* phosphorylation using protein kinase A (PKA) and [γ - 32 P]ATP. The procedures were described previously (Cheng *et al.* 1990, Denning *et al.* 1992, Gregory *et al.* 1990). Briefly, cells grown under the indicated conditions were rinsed twice with PBS (Sigma) and scraped into lysis buffer (20 mM HEPES, pH 7.0, 150 mM NaCl, 2 mM EDTA and 1% Nonidet P40) containing 1 mM β -glycerol phosphate, 1 mM L-phenylalanine, 1 mM sodium orthovanadate, 50 mM sodium fluoride and protease inhibitors (2 μ g/ml pepstatin A, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 4 μ g/ml elastinal, 0.5 mg/ml benzamidine, 1 mM phenylmethanesulfonyl fluoride, 0.4 mM iodoacetic acid, 2.5 mM phenanthroline and 0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK); all from Sigma). The cells were then homogenized in lysis buffer, placed on ice for 60 min, and then centrifuged for 30 s at 10 000 *g* in an Eppendorf benchtop centrifuge. The protein in the supernatant was quantified with BCA protein assay kit (Pierce, Rockford, IL, USA) and stored at -80°C . One milligram of lysate protein in 1 ml lysis buffer with protease inhibitors was pre-cleared with 2 μ l normal rabbit serum (used only for rabbit anti-human CFTR R domain polyclonal antibody; a gift from Dr William Guggino, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA; Zhang *et al.* 2003). 30 μ g of protein A-Sepharose (Amersham Bioscience) was added to the samples and incubated at 4°C for 2 h, all samples were then centrifuged at 10 000 *g* to remove nonspecific complexes. Subsequently, 2 μ l rabbit polyclonal anti-human CFTR R domain antibody was added and incubated at 4°C overnight. To pull down the antigen-antibody complexes, equivalent amounts of protein A beads were added to each reaction tube and incubated at 4°C for 60 min. The antigen-antibody-bead complex was precipitated with a brief spin, and then washed five times for 10 min each time with 1 ml lysis buffer. The immunoprecipitates were then washed once with 1 ml Tris-buffered saline, pH 8.0, and incubated with 5 units of the catalytic subunit of PKA (Sigma) and 10 μ Ci [γ - 32 P]ATP (NEN-Dupont) in 50 μ l PKA buffer (50 mM Tris, pH 7.5, 10 mM MgCl_2 and 0.1 mg/ml BSA) at 30°C for 1 h. Following two washes with lysis buffer, the immunoprecipitates were resuspended in 40 μ l Laemmli sample buffer (Bio-Rad) and incubated at 65°C for 4 min. The sample was spun for 2 min at 8000 *g* and the supernatant was either stored at -20°C overnight or loaded directly onto a gel. The proteins were

separated on 5% SDS/polyacrylamide gels (Bio-Rad) and prepared for autoradiography. Exposure time was 30–60 min at -70°C .

Western blotting

Immunoprecipitation and Western blot analysis were performed for CFTR protein analysis in CaCo-2 cells. After 24 h with or without stimulation with ANP, cellular protein lysates were obtained and then 200 μ g total proteins were incubated with a rabbit polyclonal anti-human CFTR R domain antibody (1:1000). The antibody was generated to peptide IEEDSDEPLERRLSLVPDSEQGE, kindly provided by Dr William Guggino (Zhang *et al.* 2003). Immunoprecipitates were collected with protein A-agarose.

After separating on 7.5% SDS/polyacrylamide gels, proteins were transferred to PVDF membrane (Bio-Rad) in Tris/glycine transfer buffer (Bio-Rad) containing 10% methanol. The membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS) for 1 h at room temperature and then was incubated overnight with primary antibodies in the blocking buffer. The primary antibody, polyclonal rabbit anti-human CFTR R domain, was diluted 1:1000 for CFTR protein. The membrane was washed three times with TTBS for 10 min each wash and incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit antibody diluted 1:5000. After washing, the blots were visualized using a standard ECL kit (Amersham Bioscience).

Transient transfections

The CFTR-promoter reporter plasmid vector construct contained the region from position -2247 to position $+52$ of the CFTR gene. The plasmid construct was kindly provided by Dr Kelly Mayo, Department of Biochemistry, Northwestern University, Evanston, IL, USA (McDonald *et al.* 1995). This location contains the major transcription start sites of CFTR (Trapnell *et al.* 1991).

The CaCo-2 cell line was grown to confluence and on the day before transfection cells were detached by exposure to 0.05% trypsin in Ca^{2+} - and Mg^{2+} -free solution, and reseeded in six-well plates to be 90% confluent on the day of transfection. The cells were transiently cotransfected with 1.5 μ g CFTR-luc promoter construct plasmid DNA for reporter assays of luciferase and 0.5 μ g pSV- β -galactosidase plasmid (kindly provided by Dr Peter Kopp, Division of Endocrinology, Northwestern University, Chicago, IL, USA) with 6 μ l LipofectAMINE 2000 reagent (Invitrogen) diluted in 250 μ l serum- and antibiotic-free DMEM and added to cells in a final volume of 500 μ l per well. After this the cells were incubated at 37°C for 3 h with the medium mentioned above, after which this medium was removed and 2 ml

supplemented DMEM were added per well and cells were cultured for 24 h post-transfection. After this the cells were washed and treated with 10^{-9} M ANP for 24 h at 37 °C. Cells were washed twice with 3 ml PBS and then lysed in 250 µl (per well) with lysis buffer (25 mM glycylglycine (gly-gly), 15 mM MgSO_4 , 4 mM EGTA, 25% Triton X100 and 2 mM dithiothreitol) for 20 min at room temperature. Luciferase activity was quantified using 200 µl cell lysates, 100 µl D-luciferin (Molecular Probes, Eugene, OR, USA) and 100 µl luciferase assay buffer (25 mM gly-gly, 15 mM MgSO_4 , 4 mM EGTA, 15 mM KH_2PO_4 , 6 mM ATP and 3 mM dithiothreitol) per sample. Light emission was detected using a TD-20/20 Luminometer (Turner Designs, Ann Arbor, MI, USA) for 30 s at room temperature. β -Galactosidase activity, used to normalize the results, was quantified using 30 µl cell lysates, 160 µl 2-Nitrophenyl- β -D-galactopyranoside (ONPG) reagent (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 and 2 mg/ml ONPG) and 810 µl β -galactosidase buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgCl_2 and 50 mM β -mercaptoethanol) at 420 nm.

Statistical analysis

ANOVA followed by Newman-Keuls multiple-comparison test was used to compare changes in the expression levels of CFTR mRNA between more than three experimental groups. We used an unpaired Student's *t* test to compare the expression levels of protein between the two different groups in Western blotting, immunoprecipitation followed by phosphorylation and the luciferase assay in CaCo-2 cells. An unpaired Student's *t*-test was also applied to compare the expression levels of CFTR mRNA in rat proximal colon between groups. Results are presented as means $\pm$ S.E. Differences were assumed to be significant when $P \leq 0.05$.

Results

CFTR mRNA modulation by ANP in CaCo-2 cells

CFTR mRNA modulation in CaCo-2 cells was determined by RPA. Cells treated with different concentrations of ANP (10^{-8} , 10^{-7} and 10^{-6} M) showed that ANP increases CFTR mRNA expression, and at concentration of 10^{-8} M a maximum effect was reached (44% increase), when compared with the NT group ($n=4-7$, $P<0.05$; Fig. 1). The CFTR mRNA modulation was also evaluated by semi-quantitative RT-PCR on extracts from cells treated with different concentrations of ANP (10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} M). CFTR mRNA expression increased 74% ($n=8$, $P<0.001$) and 54% ($n=8$, $P<0.01$) compared with the control group when cells were treated with ANP at 10^{-9} M (maximum effect) and 10^{-8} M respectively (Fig. 2). The mRNA expression of

Figure 1 RPA for CFTR in CaCo-2 cells. (A) Representative RPA results of CFTR (247 bp) and β -actin (125 bp) mRNA expression. Bands were obtained by hybridization of CFTR or β -actin probes and 30 µg total RNA extracted from CaCo-2 cells treated with ANP peptide at different concentrations. β -Actin with less exposure to the X-ray films (6 h) was utilized to normalize the experiments (indicated by arrow at 127 bp; the upper band). (B) Graphic representation of the means $\pm$ S.E. for the ratio between densitometric values of CFTR and β -actin ($n=4-7$, $P<0.05$).

CFTR analyzed by RT-PCR in cells treated at 10^{-8} , 10^{-7} and 10^{-6} M ANP demonstrated similar results to those observed using RPA. Using RT-PCR, we could also show the presence of NPR-A, NPR-B and NPR-C mRNA in CaCo-2 cells (Fig. 3).

ANP treatment increases the mature and immature forms of CFTR in CaCo-2 cells using immunoprecipitation/ phosphorylation

The immature form of CFTR, representing CFTR located in the endoplasmic reticulum, is a core-glycosylated protein that is sensitive to endoglycosidase H (band B). The fully glycosylated, endoglycosidase H-resistant CFTR protein is about 20–30 kDa larger than the core-glycosylated CFTR, and represents the mature form of CFTR (band C) that is localized in the post-endoplasmic reticulum compartments, including the plasma membrane. The CaCo-2 cell line showed mainly the fully glycosylated (band C) form of this protein, consistent with expression of mature form of CFTR. CFTR band C had an increase of approximately 5-fold in cells treated with 10^{-9} M ANP for 24 h, compared with the NT group ($n=4$, $P<0.05$; Fig. 4). We also observed an increase in the CFTR band B, the immature form of CFTR, by approximately 4-fold in cells treated with 10^{-9} M ANP ($n=4$, $P<0.05$; Fig. 4). No other unspecific bands were observed in the blot.

CFTR protein expression modulation in CaCo-2 cells by ANP using immunoprecipitation followed by Western blot

Plasma membrane proteins were extracted from CaCo-2 cells after incubation with 10^{-9} M ANP for 24 h,

Figure 2 Examination of CFTR mRNA expression modulation by ANP in CaCo-2 cells by semi-quantitative RT-PCR. Representative 1.5% agarose gel of CFTR (268 bp) and β -actin (485 bp) genes semi-quantitative RT-PCR, and graphic representation of the means $\pm$ S.E. for the ratio between densitometric values of CFTR and β -actin ($n=5-8$, $P<0.01$). There is no statistical difference between groups with the same letters (ANOVA followed by Newman-Keuls test).

a concentration which promoted maximum modulation of CFTR mRNA (result obtained on RPA and RT-PCR experiments). Densitometric analysis of CFTR protein, obtained by immunoprecipitation followed by Western blot, demonstrated an increase of about 4-fold in CFTR protein expression in cells treated with ANP compared with NT cells ($n=4$, $P<0.01$; Fig. 5). Only band C of CFTR (the most abundant form) was detected using this technique.

CFTR gene expression modulation by cGMP action in CaCo-2 cells

In CaCo-2 cells, semi-quantitative RT-PCR was performed to evaluate CFTR mRNA modulation by cGMP (an intracellular ANP-related second messenger). CaCo-2 cells treated with cGMP (1 mM) and also cells treated with both ANP (10^{-9} M) and cGMP (1 mM) showed an increase of CFTR mRNA expression, compared with the NT group, of 93% ($n=4$, $P<0.001$) and 88% ($n=3$, $P<0.01$), respectively. No statistical difference was observed in cells treated with both ANP and 5 μ M cGMPI ($n=4$) when compared with the NT group. In cells treated with both cGMP and cGMPI or cells treated only with cGMPI there was no difference in CFTR mRNA expression when compared with the NT group ($n=4$; Fig. 6).

Modulation of CFTR chloride channel gene promoter region by ANP in CaCo-2 cells

To examine potential regulation of the CFTR promoter by ANP, a 2.2 kb fragment of the human CFTR promoter

was ligated to a luciferase reporter gene (CFTR-luc) and a luciferase assay was performed. Luciferase activity is a common way to show stimulation of a gene-promoter region (McDonald *et al.* 1995, Trapnell *et al.* 1991). In transient transfection of CaCo-2 cells with CFTR-luc we observed a 24% increase in luciferase activity when cells were treated with ANP (10^{-9} M), compared with cells transfected with the same plasmid but without ANP treatment ($n=5$, $P<0.01$). This result shows that ANP has the ability to activate, directly or indirectly, a segment of the CFTR promoter region (Fig. 7).

CFTR mRNA modulation in rats subjected to volume expansion or intravenous infusion of ANP

The CFTR mRNA modulation was also evaluated by semi-quantitative RT-PCR in rats subjected to volume expansion with 0.9% saline intravenous infusion. We showed that in rat proximal colon acute extracellular volume expansion leads to an increase of CFTR mRNA expression in 60% of control group values ($n=3$, $P<0.001$; Fig. 8A). Furthermore, rats subjected to continuous intravenous infusion of synthetic ANP showed that CFTR mRNA expression increased 50% over control group values ($n=6$, $P<0.001$; Fig. 8B).

Discussion

Ion transport is essential for intestinal function. The absorption of water in the intestine, and also in the kidney, is a process secondary to solute absorption, mainly by Na^+

Figure 3 Detection of mRNAs for natriuretic peptide receptors in CaCo-2 cells by RT-PCR. Representative 1.5% agarose gel of NPR-A (219 bp), NPR-B (155 bp), NPR-C (222 bp) and β -actin (485 bp) RT-PCR products. Lanes 1, 100 bp molecular mass ladder; lanes 2, CaCo-2 cells; lanes 3, RT(–) control.

and Cl^- , the most abundant ions in the extracellular medium. In the first portion of the intestine (the small intestine) the absorption of NaCl is approximately 90%. It is also known that about 90% of water ingested reaches this part of the gastrointestinal tract. Around 1.5 l of water reaches the distal portion of the intestine (colon) daily, and only 100 ml is excreted in the feces (Turnberg 1984). The colon has the capacity to absorb 4–6 l of fluid daily (Sanioto 1999, Johnson 1997).

In pathological cases, for example, the heat-stable *Escherichia coli* toxin leads to inhibition of Na^+ absorption, as well an increase of Cl^- secretion. The accumulation of NaCl in the intestinal lumen reduces water absorption and generates a diarrhea-inducing effect in the small intestine (Field & Semrad 1993, Rolfe 1999). Several hormones and autacoids can also alter the intestinal function through action on the expression and/or activity of ion transporters, mainly in colon (Binder & Sandle 1994). The action of hormonal secretagogues, such as aldosterone, ANP and

Figure 4 Immunoprecipitation followed by *in vitro* phosphorylation from CaCo-2 cells. (A) Representative immunoprecipitation/phosphorylation results of CFTR mature form (band C) and CFTR immature form (band B). Lane 1, NT cells; lane 2, cells treated with 10^{-9} M ANP. (B) Graphic representation of means $\pm$ s.e. for the ratio between densitometric values of CFTR immature and mature forms relative to CFTR band C ($n=4$, $P<0.05$).

autacoids, for example prostaglandins, are important for maintenance of normal intestinal tract physiology (Field & Semrad 1993).

The physiological role of CFTR in the intestinal epithelia has been more evident in studies carried out in CFTR-knockout mice. These animals, even when they are heterozygotes for mutations in the CFTR gene,

Figure 5 Detection of CFTR protein by immunoprecipitation and Western blot analysis in CaCo-2 cells. (A) Representative immunoblot for CFTR in membrane extracts prepared from CaCo-2 cells. Lane 1, NT cells; lane 2, cells treated with 10^{-9} M ANP. (B) Graphic representation of means $\pm$ s.e. for densitometric values of CFTR protein bands obtained in the Western blot experiments ($n=3$, $P<0.05$).

Figure 6 CFTR mRNA expression modulation by cGMP in CaCo-2 cells (semi-quantitative RT-PCR). Agarose gel representative of semi-quantitative RT-PCR of CFTR (268 bp) and β -actin (485 bp) genes, and graphic representation of the means $\pm$ S.E. from the ratio between densitometric values of CFTR and β -actin. Lane 1, NT cells; lane 2, cells treated with ANP; lane 3, cells treated with ANP+cGMPI; lane 4, cells treated with cGMP+cGMPI; lane 5, cells treated with cGMP; lane 6, cells treated with cGMPI; lane 7, cells treated with ANP+cGMP. ANP (10^{-9} M), cGMP (1 mM), cGMPI (5 μ M). There is no statistical difference between groups with the same letters ($n=4$, $P<0.01$; ANOVA followed by Newman-Keuls test).

do not respond completely to the effects of the secretagogues compared with normal animals (Grubb & Boucher 1999). It is a fact that most of these mice die from intestinal complications, which clearly indicate the crucial role of CFTR in these epithelia (Snouwaert *et al.* 1992, Eckman *et al.* 1995).

During extracellular volume expansion, changes in the plasma concentration of several hormones occurs. In this situation, for example, while aldosterone and angiotensin concentrations are decreased at plasma levels, the ANP concentration is increased. As has been largely reported, the intestinal tract is a target organ for ANP. ANP in the small intestine was found to reduce the reabsorption of water, Na^+ and dextrose in rats (Martinez Seeber *et al.* 1986). In dogs, Matsushita *et al.* (1991) demonstrated that ANP suppressed the reabsorption not only of water and Na^+ , but also Cl^- across the jejunum. In the large intestine

ANP causes a transient increase in potential differences and a short-circuit current across the proximal and distal colon mounted in Ussing chambers (Argenzio & Armstrong 1993, Moriarty *et al.* 1990, Vaandrager *et al.* 1992), suggesting ANP's involvement in ion transport in this tissue. However, these changes were not observed in the human colonic cell line T84 (human colonic carcinoma derived from metastasis in the lung), which has different characteristics to CaCo-2 cells.

Acting directly or indirectly it is suggested that the hormone-sensitive chloride channels at the apical membranes of epithelial cells provide well-characterized mechanisms that control epithelial cell conductance and, because it was shown previously that ANP's effects in rat colon are both chloride-dependent and sensitive to chloride-channel blockers, this channel is probably one of ANP's ultimate molecular targets in the colon (Moriarty *et al.* 1990). CFTR could be that chloride channel once it is expressed in colon, and could control the conductance of other ions.

In our experiments, the animals subjected to extracellular volume expansion showed a 60% increase of CFTR mRNA expression in proximal colon compared with control animals. This increase was probably related to the ANP plasma concentration enhancement, as we also observed a similar rate of increase (50% of CFTR mRNA expression) in proximal colon of rats subjected to continuous intravenous infusion with synthetic ANP.

In vitro, using CaCo-2 cells as a model, ANP is able to increase the expression of the CFTR mRNA with maximum stimulation observed at 10^{-9} M, the physiological basal plasma level of ANP found in humans (Ando *et al.* 1990, Sagnella 1998). This stimulation was statistically similar to cells treated with ANP at 10^{-8} , 10^{-7} and 10^{-6} M, which is the same pattern observed in other

Figure 7 Modulation of CFTR chloride-channel gene-promoter region in CaCo-2 cells by ANP. Shows the luciferase activities in CaCo-2 for the NT group and cells treated with 10^{-9} M ANP. Luciferase activities were normalized by cotransfection with the pSV- β -galactosidase plasmid. Means $\pm$ S.E. are shown ($n=4$, $P<0.01$).

Figure 8 CFTR mRNA expression modulation in proximal colon of rats subjected to extracellular volume expansion or ANP intravenous infusion. Agarose gel representative of semi-quantitative RT-PCR of CFTR (615 bp) and GAPDH (211 bp) genes. Graphical representation of means $\pm$ S.E. from the ratio between densitometric values of CFTR and GAPDH. (A) Animals subjected to extracellular volume expansion (VE) and control group (Control; $n=3$, $P<0.0001$). (B) Animals treated with synthetic ANP (ANP) and control group ($n=6$, $P<0.0001$). MW: molecular mass ladder; RT(-): negative control without RT.

studies such as for the heme oxygenase-1 (HO-1) gene (Kiemer *et al.* 2003).

In CaCo-2 cells we observed that CFTR mRNA expression was increased 74% when compared with NT cells. In the same way CFTR protein was found to be increased in cells treated with 10^{-9} M ANP of approximately 5-fold for the mature form of CFTR (functional protein) and approximately 4-fold for the immature form of CFTR protein (non-functional protein). The greater stimulation at the protein level compared with the mRNA level suggests a possible post-transcriptional action of ANP.

It is important to mention that ANP's half-life is 2–5 min, but this action leads to several changes at the cellular level (second messengers, expression of transcriptional factors, etc.) that could change gene expression at both the mRNA and protein levels (Wolf *et al.* 1995). Three receptors are related to different natriuretic peptides. NPR-A binds ANP with specificity and high affinity. It was suggested that NPR-B may be relatively more selective for brain natriuretic peptide than for ANP when compared with NPR-A (Takayanagi *et al.* 1987). The NPR-C binds ANP with high affinity, and it is involved in the sequestration and metabolic clearance of ANP. It was suggested that NPR-C is coupled to the adenylate cyclase/cAMP signal transduction pathways (Anand-Srivastava & Trachte 1993). In our results we showed high abundance of NPR-A and NPR-B in CaCo-2 cells but the involvement of each receptor in the CFTR expression stimulation must still be clarified. The ANP binding to NPR-A and NPR-B produces an increase in cGMP in several types of tissue, including colonic cells. It was shown previously that the enhanced cGMP in cells induces phosphorylation of different proteins

that can lead to a stimulation or inhibition of activity (Gonzalez Bosc *et al.* 2000, Lin *et al.* 1992). In addition, it was shown that cGMP is involved in control of gene expression via its action on different gene-promoter regions or by controlling the activities of transcription factors such as AP-1, c-Jun N-terminal kinase and extracellular-signal-regulated kinase (Kiemer *et al.* 2003, Pilz & Casteel 2003). Acting in these two ways the cGMP could control gene expression. Our results showed that cGMP increases CFTR mRNA expression in CaCo-2 cells, in the same manner observed when ANP is used without cGMP addition. Furthermore, ANP and cGMP together did not have cumulative effects on CFTR mRNA expression. These results suggest that ANP acts via cGMP in CaCo-2 cells, leading to the modulation of CFTR. On the other hand, we cannot discard the possibility that cGMP might be acting through the inhibition of phosphodiesterases, permitting an increase of cAMP levels, the second messenger that is also capable of activating CFTR chloride-channel conductance and its gene expression (McDonald *et al.* 1995), enhancing the action of cGMP. Experiments are necessary to clarify the functional significance of the stimulation of expression CFTR by ANP.

The *in vitro* model also helped to observe that ANP stimulates the CFTR promoter region, suggesting that the modulation of CFTR expression through ANP action in CaCo-2 cells could be secondary to the increase of gene transcription. However, the post-transcriptional regulatory effects of this hormone cannot be discarded.

In conclusion, the present study is the first to directly access the effect of ANP on CFTR expression in proximal colon of rats and in CaCo-2 cells suggesting that this

peptide has a physiological importance in ion transport in the gastrointestinal tract.

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